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Award Number: W81XWH-09-2-0080

TITLE: Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

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REPORT DATE: September 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 SEP 2010 - 31 AUG 2011	
4. TITLE AND SUBTITLE Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-2-0080	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Thomas Neylan E-Mail: mindy.sivasubramanian@va.gov				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northern California Institute for Research San Francisco, CA 94121				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT During Year 1, progress was made in preparation for the initiation of subject enrollment. The study protocol has been finalized and a comprehensive assessment battery incorporating neurocognitive, objective alertness, and subjective symptom assessments has been assembled and finalized. The testing battery was selected on the basis of sensitivity to sleep-inducing agents and military relevance. Study equipment and supplies have been purchased. Scientific and human use approvals have been solicited. Study documentation has been submitted to the appropriate Institutional Review Boards for approval, and an Investigational New Drug application (IND) has been filed with the Food and Drug Administration. An IND number has been assigned which will allow for interstate shipment of study drug. In accordance with relevant federal regulations, the study has been registered with clinicaltrials.gov. Key study personnel have been hired and trained in preparation for upcoming enrollment. As a result of the progress made during Year 1, subject recruitment and enrollment will begin during the early months of Year 2.					
15. SUBJECT TERMS Neurocognitive Performance, Sleep, Hypocretin, Orexin					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 88	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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ANNUAL PROGRESS REPORT

Year 2

September 30, 2011

Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

USAMRMC Grant W81XWH-09-2-0080

Thomas Neylan, M.D., Initiating Principal Investigator

INTRODUCTION

An integrated translational study will be conducted to examine the effect of a novel hypocretin/orexin antagonist, almoxant (ALM), compared to a standard hypnotic, zolpidem (ZOL), and placebo (PBO) on neurocognitive performance at peak concentration post dosing. The human study component (Task 1; responsible individual: Thomas Neylan, M.D.) will establish whether ALM is superior to ZOL in relation to neurocognitive side effects. It is hypothesized that healthy human subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance compared to subjects receiving 100mg or 200mg doses of almoxant or placebo. Study subjects (n=200) will receive a randomly assigned, one-time dose of study drug in an inpatient hospital setting. A battery of neurocognitive, objective alertness, and subjective symptom assessments will be administered prior to and following dosing. Assessments to be administered were selected based upon their demonstrated sensitivity to sleep-inducing agents and their military relevance. The animal study component (Tasks 2 – 5; responsible individual: Thomas Kilduff, Ph.D.) will compare the neural circuitry that underlies the activity of the abovementioned compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Progress associated with each task outlined in the approved Statement of Work is listed below:

Task 1: *Test the hypothesis that healthy human subjects receiving ZOL 10mg will show greater impairment in neurocognitive performance compared to subjects receiving PBO or the 2 doses (100mg, 200mg) of ALM.*

The Task 1 subtasks listed below have been completed prior to or during Year 2:

Subtask #1: Write Protocol

The study protocol was finalized during Year 1, and modifications were made to the protocol during Year 2. The neuropsychological testing battery was also tested and fine-tuned prior to the initiation of enrollment. The current version of the protocol and the final neuropsychological testing battery are included in Appendix 1.

Subtask #2: Obtain Scientific and Human Use Approvals

Study documentation was submitted to the appropriate Institutional Review Boards (IRBs) and the Food and Drug Administration (FDA) for approval prior to the end of Year 1. All human subjects approvals were obtained during Year 2. Approval timelines are detailed below:

- IRB Approval:

- **Initial Approval**

The University of California, San Francisco Committee on Human Research (UCSF CHR) provided initial approval on October 29, 2010. The Department of Veterans Affairs Medical Center Research and Development Committee (VA R&D Committee) provided approval on January 6, 2011. The U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO) provided initial approval on March 9, 2011.

Approval of Amendments

An amended Investigator's Brochure was provided by Actelion Pharmaceuticals on March 23, 2011 which necessitated revisions to the study protocol and informed consent document. Enrollment could not begin until all Institutional Review Boards approved the revised study documents. The UCSF CHR and the VA R&D Committee approved the revisions on May 3, 2011. The USAMRMC HRPO approved the revisions on May 10, 2011, at which point enrollment could be initiated.

Continuing Review

An annual continuing review application was approved by the UCSF CHR on September 20, 2011, extending the study's approval expiration to October 4, 2012. Continuing review approval from the VA R&D Committee is expected to be received by October 1, 2011. All continuing review approvals will be submitted to a continuing review analyst at the USAMRMC HRPO once received.

- Investigational New Drug Application (IND): At the conclusion of Year 1, an IND application was filed with the FDA in order to obtain approval to receive study drug from Actelion Pharmaceuticals. The IND went into effect on October 21, 2010. An amended study protocol was submitted to the FDA on May 18, 2011 following revisions in response to the Investigator's Brochure Amendment received from Actelion Pharmaceuticals in March, 2011. In accordance with FDA requirements, an annual progress report will be submitted by no later than December 20, 2011.

Subtask #3: Purchase Study Related Equipment/Supplies

The majority of study related equipment (including sleep equipment, actigraphs, psychomotor vigilance tests, and neuropsychological testing supplies) was purchased and tested during Year 1. Further testing and piloting of the equipment was performed during the early part of Year 2. All remaining study supplies were purchased and tested during Year 2.

Study drug (provided by Actelion Pharmaceuticals) arrived onsite at the UCSF Medical Center pharmacy in March, 2011. An external unblinded monitor has been appointed to perform regular drug accountability checks to confirm that drug is stored properly and in accordance with expiration dates.

Subtask #4: Train Laboratory Personnel

The majority of study personnel was hired and trained during Year 1. However, the following positions were filled during the early part of Year 2:

- **Recruiter:** The Recruiter was hired in Y2, Q2 and has been responsible for all subject recruitment and outreach efforts including internet postings, flyers, etc. The Recruiter has conducted telephone assessments to determine eligibility, scheduled consent appointments and initial diagnostic evaluation interviews, and maintained a participant tracking database.
- **Research Assistant:** The Research Assistant was hired in Y2, Q2 and has provided assistance to the Study Coordinator and Sleep Technician to support the daily operational activities of the study. She has worked directly with participants to coordinate their appointments for physical exams and blood draws. The Research Assistant has been trained in the sleep polysomnography procedures, and has helped to maintain the polysomnography and actigraphy equipment. She has also been responsible for the preparation of clinician interview materials and self-report questionnaires.

Subtask #5: Collect Data on 200 Volunteers

Recruitment and enrollment efforts were initiated in May 2011 (Y2, Q3), following receipt of all regulatory approvals. Enrollment details and future plans are outlined below:

A.) Enrollment Progress During Year 2

- **Recruitment:** Recruitment advertising efforts have involved monthly postings on the internet. These ads have generated a substantial response rate, as approximately 600 individuals have shown an interest in the study. 386 interested participants were pre-screened by phone prior to being scheduled for full eligibility assessments.
- **Screening:** 34 participants met phone pre-screen requirements and were invited to take part in an informed consent visit. After obtaining informed consent, eligibility screening procedures were conducted at the San Francisco VA Medical Center. The eligibility screening procedures include a mental health screening, self-report questionnaires related to caffeine use, tobacco use, alcohol use, and sleep habits, a physical exam, urine drug and pregnancy screen, and blood draw for hematology and serum chemistry panels.
- **Eligible Participants:** 21 participants have been identified as eligible. 11 of those participants have completed all study procedures and 1 participant dropped out of the study early due to an unforeseen scheduling conflict. The remaining 9 eligible participants are scheduled to complete study procedures within 30 days of the submission of this report.

B.) Enrollment Challenges Faced During Year 2:

Enrollment Delays

The Investigator's Brochure Amendment which was issued by Actelion Pharmaceuticals in March, 2011 delayed the planned initiation of enrollment. In response to the amendment, the study protocol and informed consent document needed to be revised, and those revisions required approval from the UCSF CHR, the VA R&D Committee, and the

USAMRMC HRPO prior to the initiation of enrollment activities. The IB Amendment caused a delay of approximately 8 weeks.

- **Recruitment Challenges**

Recruitment ads have been posted on craigslist.org on a monthly basis since May, 2011, and each ad typically generates over 100 responses. Interested respondents are contacted by the study recruiter for a brief phone pre-screen before being scheduled for an informed consent meeting with the Study Coordinator and the next phase of eligibility procedures. Thus far, only 9% of phone screened participants have met the requirements necessary to move forward with consent meetings and full eligibility procedures. Recruitment metrics suggest that 30% of participants are ruled out during phone pre-screens because they have sleep problems, while 20% of participants are ruled out due to medical problems. Another 15% are ruled out due to excess alcohol, caffeine, or drug use. This knowledge has helped the study staff to create revised recruitment materials and outreach strategies designed to more accurately target the intended population of healthy volunteers. This effort should ultimately produce a more efficient yield of participants from phone screens, therefore increasing the total number of screened and enrolled participants each month.

C.) Future Enrollment Strategies:

- **Recruitment and Outreach:**

Revised recruitment materials were recently submitted to the UCSF CHR and were approved on September 20, 2011. The revised recruitment materials emphasize that healthy volunteers are needed for the study and that participants must have healthy sleep patterns. It is anticipated that this will eliminate the number of responders who think that the study is for the treatment of insomnia. Outreach efforts planned for the immediate future include the creation of posters, flyers, and brochures which will be distributed on the campuses of local universities. The study team believes that this will accurately target the intended population.

- **Monthly Enrollment Projections:**

In order to enroll 200 participants by Q2, Y4, the quarterly enrollment target of 22 participants listed in the Statement of Work will need to increase to 33 quarterly participants, or 11 participants per month. The study team has put the necessary staff, equipment, and facilities in place to enroll and randomize two participants simultaneously. This capability will allow the study team to do its best to reach the quarterly enrollment goal of 33 participants going forward.

Subtask #6: Score and Analyze Data

Study data has been scored and cleaned on an ongoing basis since the initiation of enrollment to shorten the cleaning and analysis timelines required during Y4. Specific progress made during Y2 is listed below:

- Study Database and Data Entry: The study database was built and tested shortly after the first subject completed the study. As a result, data entry has been taking place on an ongoing basis and remains up to date.

- Data Cleaning: All data is QC'd and scored by trained and qualified study staff prior to being entered into the study database. Additionally, edit checks have been programmed within the study database which generate queries for the Study Coordinator to address on an ongoing basis.

Other Accomplishments Completed During Year 2:

- Reporting:
Ongoing reports have been submitted as follows:
 - Safety listings are submitted to Actelion Pharmaceuticals on a monthly basis.
 - Progress reports are submitted to the Department of Defense on a quarterly basis.
 - Progress reports are submitted to the FDA on an annual basis.
- Human and Animal Study Collaboration:
The San Francisco (human study) and SRI International (animal study) teams met monthly via teleconference throughout Year 2 to share progress updates, scientific rationale, and future planning initiatives. The annual investigator/collaborator meeting was hosted by SRI in August, 2011. Members from each team gave presentations related to research rationale, progress, and future directions.

Tasks 2 – 5: Please refer to the attached report from Dr. Kilduff (Appendix 2) which details the progress made in reference to the animal studies. The Statement of Work for the animal component of the research project has been revised and is attached in Appendix 3.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 Accomplishments:

- The study protocol has been amended.
- Human subjects approvals have been obtained from the UCSF CHR, the VA R&D Committee, and the USAMRMC HRPO.
- All remaining study personnel have been hired and trained on the study protocol and procedures.
- All remaining study equipment and supplies have been purchased and tested. Study drug has been provided by Actelion Pharmaceuticals.
- 19 eligible participants have been identified through recruitment and screening efforts.
- Recruitment materials have been revised and outreach strategies have been developed to increase enrollment rates to 11 eligible participants per month.

Tasks 2 – 5 Accomplishments:

Please refer to the attached progress report from Dr. Kilduff (Appendix 2).

REPORTABLE OUTCOMES

Reportable outcomes related to Task 1 will not be available until Year 4. Reportable outcomes related to Tasks 2 – 5 are noted in the attached progress report from Dr. Kilduff (Appendix 2).

CONCLUSION

Preclinical data indicate that animals treated with almorexant are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The purpose of this research is to test related hypotheses in both animals and humans. Enrollment of human subjects began during Year 2 and is expected to continue until the middle of Year 4. The Year 2 findings from the animal component of the study were consistent with the hypothesis that disfacilitation of wake-promoting systems by almorexant results in less functional impairment than the general inhibition of neural activity produced by zolpidem (Appendix 2).

APPENDICES

Appendix 1: Human Study Protocol and Final Neuropsychological Testing Battery

Appendix 2: Animal Studies Progress Report

Appendix 3: Revised Statement of Work

Appendix 1: Human Study Protocol and Neuropsychological Testing Schedule

CLINICAL STUDY PROTOCOL

Title: Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

Protocol number: NEY-1413

Protocol Version/Date: Final Version 7.0 15 August 2011

Phase: Investigator-Initiated

Investigational Drug: Almorexant

Investigator-Sponsor: Thomas C. Neylan, M.D.
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Medical Monitor: Frank Schoenfeld, MD
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Study Sites: University of California, San Francisco
Clinical and Translational Sciences Institute
Clinical Research Center
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San Francisco, CA 94143

San Francisco Department of Veterans Affairs Medical
Center
4150 Clement Street
San Francisco, CA 94121

This clinical study will be conducted in accordance with Standard Operating Procedures (SOPs), current Good Clinical Practice (GCP) and the provisions of International Conference on Harmonization (ICH) Guidelines

Protocol Approval
NEY-1413
Final Version 6.0 04 April 2011

Investigator-Sponsor		
	Thomas Neylan, MD	Date
Co-Investigator		
	Steven Batki, MD	Date
Co-Investigator		
	Kristin Samuelson, Ph.D.	Date
Biostatistician		
	Thomas Metzler, M.S.	Date
Study Coordinator		
	Mindy Sivasubramanian, M.S.	Date

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ABBREVIATIONS

AE	Adverse Event
AASM	American Academy of Sleep Medicine
BzRAs	Benzodiazepine Receptor Agonists
CRC	Clinical Research Center
CCRC	University of California, San Francisco Clinical Translational and Sciences Institute Inpatient Clinical Research Center
UCSF CHR	University of California, San Francisco Committee on Human Research
CNS	Central Nervous System
CPT	Conners' Continuous Performance Test II
CRF	Case Report Form
DMP	Data Management Plan
DS	Digit Span Subtest of the Wechsler Adult Intelligence Scale Fourth Edition
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders Fourth Edition Text Revision
EEG	Electroencephalogram
FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric Acid
GCP	Good Clinical Practice
GP	Grooved Pegboard Motor Test
ICH	International Conference on Harmonisation
IND	Investigational New Drug Application
IQ	Intelligence Quotient
IRB	Institutional Review Board
MWT	Maintenance of Wakefulness Test
NREM	Non-Rapid Eye Movement
ORP HRPO	Federal Office of Research Protections Human Research Protection Office
P-A	Paired Associates Learning Task
PSG	Polysomnography
PSQI	Pittsburgh Sleep Quality Index
PVT	Psychomotor Vigilance Test
QC	Quality Control
R&D Committee	Veterans Affairs Research and Development Committee

REM	Rapid Eye Movement
RAVLT	Rey Auditory Verbal Learning Test
SAE	Serious Adverse Event
SC	Symptom Checklist
SCID	Structured Clinical Interview for DSM-IV TR Axis I Disorders
SFDVAMC	San Francisco Department of Veterans Affairs Medical Center
SSS	Stanford Sleepiness Scale
Stroop	Stroop Color-Word Test
Towers	Tower Test from Delis-Kaplan Executive Function System
USAMRMC	U.S. Army Medical Research Materiel Command
WAIS-IV	Wechsler Adult Intelligence Scale Fourth Edition
WASO	Wake after Sleep Onset
WMS	Wechsler Memory Scale

SYNOPSIS

Protocol Number:	NEY-1413
Study Title:	A Double-Blind, Placebo-Controlled, Randomized, Parallel-Group Study Comparing the Effect of a Novel Hypocretin/Orexin Antagonist (Almorexant) Versus a Standard Hypnotic (Zolpidem) and Placebo on Neurocognitive Performance
Number of Sites:	1
Treatment Duration:	One-time Dose
Study Duration:	10 days, with a follow-up visit within 5 – 12 days of dosing
Study Population:	216 healthy male and female volunteers
Rationale:	In recent years, there has been increased focus on neurocognitive effects of hypnotic medications that adversely affect behavior during unanticipated awakenings during the night. Concerns regarding untoward effects of hypnotics during the sleep period have led to a Food and Drug Administration (FDA) class warning for all hypnotic drugs. These concerns are particularly relevant to the personnel of the military and those in other professions who have an occupational risk of poor sleep and who are expected to perform without impairment upon awakening. Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with almorexant are easily aroused from sleep and behave free of ataxia and other impairment. If this observation is confirmed in humans, it will have substantial implications for the management of disturbed sleep in both military and civilian populations.
Study Objectives:	To compare neurocognitive performance at peak concentration at midpoint during the habitual wake period in subjects randomized to almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo.
Study Design:	The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers and who are free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been

	<p>confirmed, subjects will be scheduled for the 10-day study period. During the first seven days of the study period (the sleep/wake monitoring period), subjects will be asked to maintain a sleep diary and wear a wrist activity monitor (actigraph) 24 hours per day. Subjects will be admitted to the CCRC on the eighth day of the study period, two days prior to study drug administration. Subjects' sleep will be monitored with polysomnography (PSG) during each night on the CCRC, and subjects will continue to maintain a sleep diary during the three-day hospital stay. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). Study drug will be provided to a nurse on the CCRC by an unblinded research pharmacist. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from CCRC on the morning of the fourth day on the unit. They will be required to return to the CRC at the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).</p>
Inclusion Criteria:	<ol style="list-style-type: none"> 1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments; 2.) Habitual wake time between 0600 hr and 0800 hr maintained within the past month; 3.) Habitual bedtime between 2200 hr and 0000 hr maintained within the past month; 4.) Body Mass Index (BMI) >18 and $< 28 \text{ kg/m}^2$; 5.) Ability to communicate well with the Investigator and to understand the study requirements.
Exclusion Criteria:	<ol style="list-style-type: none"> 1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI); 2.) Current presence of two or more risk categories on the Berlin Questionnaire for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea. 3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic

	<p>stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of depressive disorder not otherwise specified, assessed using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) Axis I Disorders (SCID);</p> <p>4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID;</p> <p>5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study;</p> <p>6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for ≥ 10 minutes which resulted in the development of persistent symptoms lasting ≥ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent.</p> <p>7.) Systemic illness affecting central nervous system (CNS) function;</p> <p>8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, history of myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia;</p> <p>9.) Asthma or other reactive airway diseases;</p> <p>10.) Hepatic impairment (Child-Pugh A, B, C);</p> <p>11.) Any other chronic or unstable medical conditions;</p> <p>12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor;</p> <p>13.) Treatment with another investigational drug;</p> <p>14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor;</p> <p>15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization;</p> <p>16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization;</p> <p>17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day;</p> <p>18.) Self-reported consumption of alcohol within the past 30 days of > 14 standard drinks per week or ≥ 5 standard drinks</p>
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	<p>on any day (men), or > 7 standard drinks per week or ≥ 4 standard drinks on any day (women).</p> <p>19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen;</p> <p>20.) Known liver disease or abnormal liver function tests assessed at the time of screening;</p> <p>21.) Self-reported regular caffeine use in excess of 200 mg per day on average within six months of screening;</p> <p>22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours);</p> <p>23.) Shift work within one month prior to the screening visit or planned shift work during the study;</p> <p>24.) Subjects who have traveled > 3 time zones within one week prior to the screening visit or any other visit;</p> <p>25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.</p>
Outcome Measures:	<p><u>Primary Endpoints:</u></p> <ol style="list-style-type: none"> 1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test, Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT). 2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose. 3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs. <p><u>Secondary Endpoints:</u></p> <ol style="list-style-type: none"> 1.) A comparison between groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose. 2.) A comparison between groups on Stanford Sleepiness Scale (SSS) scores. <p><u>Covariates:</u></p> <ol style="list-style-type: none"> 1.) Polysomnography (PSG) – Total Sleep Time on the night prior to the day of dosing. 2.) Actigraphy – Average sleep duration.
Statistical	It is hypothesized that subjects receiving zolpidem 10mg will show

Considerations:	<p>greater impairment in neurocognitive performance compared to subjects receiving placebo, almorexant 100mg, or almorexant 200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. Where multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (i.e., pre- vs. post-medication) interaction effect serving as the test of the hypothesis. Where a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Planned comparisons will be conducted to compare the zolpidem 10mg group with placebo, almorexant 100mg, and almorexant 200mg separately. P-value adjustments will be made for multiple endpoint variables within any given neurocognitive domain using a step-down non-parametric re-sampling-based procedure. Primary analyses will be intent-to-treat, including all subjects randomized regardless of dropout or missing data status. Missing data will be carefully characterized and multiply imputed if necessary.</p>
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1. INTRODUCTION

1.1 Background

In recent years, there has been increased focus on cognitive side effects associated with sleep-inducing medications that may contribute to unusual behavior during unexpected awakenings during the night. Concerns regarding these side effects have led to a Food and Drug Administration (FDA) class warning for all sleep-inducing medication. These concerns are particularly important to the military and other professions that have an occupational risk of poor sleep and being unexpectedly awakened with an expectation to perform without impairment.

Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Hypocretin/orexin is a neuropeptide system that stimulates arousal and is involved in sleep regulation. Disruption of the hypocretin/orexin system has been shown to result in the sleep disorder narcolepsy in both animals and humans, indicating that this system is part of the intricate sleep/wakefulness regulatory network. Hypocretin receptors are found in many brain regions, although receptor expression is weak in the cortex and high in brain regions associated with arousal state regulation, particularly the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems. Since the hypocretin peptides are excitatory throughout the brain, hypocretin antagonists work by blocking this excitation rather than producing a generalized inhibition. To the contrary, benzodiazepine receptor agonists (BzRAs) such as zolpidem affect gamma-aminobutyric acid (GABA_A) receptors which have widespread distribution in the central nervous system (CNS), particularly in the cerebral cortex. BzRAs therefore cause a general inhibition of neural activity (2).

1.1.1 Preclinical Background

Preclinical data demonstrate that almorexant produces a profile that is unique among currently marketed hypnotic medications. For example, preliminary study results in rats treated with one of three doses (10mg/kg, 30mg/kg, and 100mg/kg) of almorexant, zolpidem or placebo in the middle of the dark active period (six hours after lights offset) demonstrated that the 30mg/kg and 100mg/kg doses of almorexant and zolpidem increased non-rapid eye movement (NREM) sleep for several hours after dosing, whereas 10mg/kg of almorexant had a more transient effect. All three doses of almorexant increased rapid eye movement (REM) sleep while REM was suppressed by zolpidem. Consequently, the REM-NREM ratio was unchanged relative to vehicle in animals treated with almorexant, but zolpidem produced a decreased REM-NREM ratio which is characteristic of BzRAs. When cumulative effects were assessed over the entire six-hour post-treatment period, it was evident that almorexant produced a dose-dependent decrease of wake and a dose-dependent increase in both NREM and REM sleep. This profile of a proportional increase of REM and NREM sleep appears to be unique among currently marketed hypnotic medications (3).

Additionally, almorexant appears to have few side-effects on regulated physiological systems. Preliminary studies comparing the effects of varying doses of almorexant, zolpidem, and placebo on core body temperature in rats revealed that zolpidem-treated

animals experienced a significant and prolonged change in core body temperature post-treatment, but there was relatively little change in core body temperature associated with any dose of almorexant (3).

In studies involving somnolent rats treated with almorexant, the rats showed an immediate reversibility of the hypnotic effect with no impairment on motor performance tasks (3). If similar observations are confirmed in humans, there will be enormous implications for the management of disturbed sleep in both military and civilian populations.

1.1.2 Clinical Background

Because hypocretins are implicated in coordinating states of wakeful vigilance, there has been a rapid development of small molecule hypocretin 1 and hypocretin 2 antagonists for possible use in insomnia. At present, there are robust drug discovery programs for hypocretin1/hypocretin 2 antagonists sponsored by Actelion, Glaxo-Smith Kline, Merck, Banyu, Sanofi-Aventis, and Janssen. In 2007, Actelion presented results of a multi-site, double-blind placebo controlled trial in insomnia patients examining the effects of 50mg, 100mg, 200mg, and 400mg doses of almorexant at bedtime. The results showed significant improvement in sleep efficiency and reduced wake after sleep onset (WASO) at doses of 100mg and higher (4). There was no occurrence of cataplexy at any of the dosages used. Almorexant has an elimination half-life of 1.4 hours and effects on sleep electroencephalography (EEG) were absent after 6.5 hours (3).

Almorexant was well-tolerated in studies completed to date, including nineteen Phase I studies in healthy and hepatically impaired subjects, two dose-finding studies in adult and elderly patients with primary insomnia, and one Phase III study in primary insomnia. 519 healthy and hepatically impaired subjects were exposed to at least one dose of almorexant in Phase I studies. 633 subjects with primary insomnia have been exposed to at least one dose of almorexant in completed studies. Maximum exposure was up to 400mg daily for 1 day or up to 200mg for 16 days. 166 patients with primary insomnia received 200mg for at least 14 days, and 176 received 100mg for at least 14 days. The most frequently reported adverse events with almorexant were headache, fatigue, dizziness, and somnolence (40).

1.2 Rationale

At appropriate doses, all currently available FDA-approved prescription sleep-inducing agents induce restorative sleep. However, they also exert substantial performance-impairing effects at peak concentration in multiple domains of neurocognitive function. For example, multiple studies have shown impairment in driving within the six-hour window after ingesting zolpidem (6, 7). Other studies have documented impairment in balance and postural tone within two hours of taking zolpidem (8). Furthermore, there is solid evidence that at peak concentration, currently available sleep-inducing agents significantly impair the ability to consolidate new memories (9-12). This evidence therefore precludes the use of sleep-inducing agents under operational conditions in which individuals might be called upon to perform without impairment after taking the

agent, which is particularly relevant to populations involved in military combat. Further, there is an enormous accumulation of data linking disturbed sleep to a wide range of outcomes including daytime fatigue (13-15), impaired concentration and attention (16-19), increased risk for accidents and injuries (20, 21), worsened quality of life (22), increased aggression (23-26), and increased use of alcohol (27, 28). Several studies have also demonstrated that disturbed sleep is a potent risk factor for later onset development of major depression, panic disorder, alcohol, and substance abuse (27-30). Therefore, an effective treatment for sleep disturbances that can be safely utilized in deployed military personnel in combat operations without performance-impairing effects has the potential for improving the success of combat operations, inoculating soldiers against battlefield stress-related psychiatric illnesses, and preserving the psychological health of the soldiers throughout the full deployment lifecycle. The availability of such a treatment would also have a positive impact on the overall quality of life, physical, and psychological well being of the civilian population.

The study discussed in this protocol will involve a double-blind, placebo-controlled, randomized, parallel-groups study design and will involve a one-time oral administration of one of four dosing options to healthy volunteers: almorexant 100mg, almorexant 200mg, zolpidem 10mg, and placebo. These dosages have demonstrated favorable safety profiles in clinical trials (5). Subjects will be dosed at the average midpoint of the habitual wake period. Neurocognitive performance assessments will be administered at the time of peak plasma concentration. The study will establish whether almorexant is superior to zolpidem and placebo regarding neurocognitive performance at the estimated peak plasma concentration.

2. CLINICAL STUDY OBJECTIVES

2.1 Primary Objectives

Primary endpoints are listed below:

- 1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test (GP), Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT).
- 2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose.
- 3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs.

2.2 Secondary Objectives

Secondary endpoints are listed below:

- 1.) A comparison between dosing groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose.
- 2.) A comparison between dosing groups on Stanford Sleepiness Scale (SSS) scores.

The following outcomes will be analyzed as covariates:

- 1.) Polysomnography (PSG) - Total Sleep Time on the night prior to the day of dosing.
- 2.) Actigraphy – Average sleep duration.

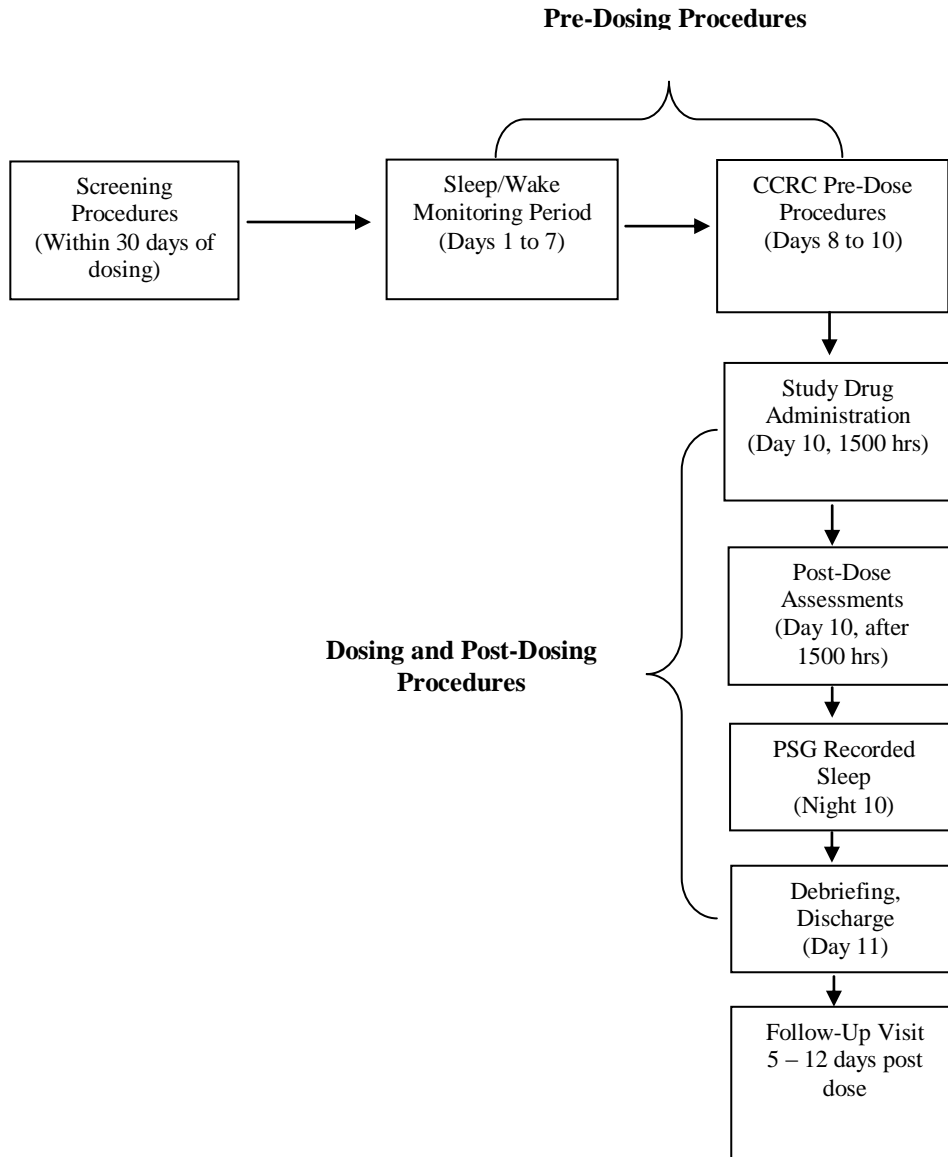
3. STUDY DESIGN

The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers (1) as listed below:

- 1.) Subject has no complaints of sleep disturbance or daytime symptoms attributable to unsatisfactory sleep.
- 2.) Subject has a routine sleep/wake schedule characterized by regular bedtimes and rising times.
- 3.) There is no evidence of a sleep-disruptive medical or mental disorder.
- 4.) There is no evidence of sleep disruption due to a substance exposure, use, abuse, or withdrawal.
- 5.) There is no evidence of a primary sleep disorder.

Subjects will also be free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been confirmed, subjects will be instrumented with wrist actigraphs to record their sleep/wake patterns for seven days; subjects will also be asked to complete a sleep diary during this one-week time period. Subjects will be admitted to the CCRC on the day after completion of the one-week sleep/wake monitoring period and two days prior to drug administration. Subjects' sleep will be monitored with PSG during each night at the CCRC, and sleep apnea will be screened for during the first night of PSG. Subjects will continue to maintain a sleep diary while at the CCRC. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). An unblinded research pharmacist will provide study drug to a nurse on the CCRC for dispensing. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered at regular intervals for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from the CCRC during the morning of the fourth day on the unit. They will be required to return to the CRC at the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).

3.1 Study Design Schematic



4. SUBJECT SELECTION

Medically healthy men and women ages 19-39 (N = 216) will be recruited from newspaper advertisements, web based postings, websites, and flyers posted in various university and community sites. The age range is restricted to an upper limit of 39 years as a result of research showing a change in middle-aged individuals (defined as 40+ years of age) in terms of total sleep time and other sleep parameters that can affect performance outcomes independent of sleep deprivation and/or drug administration, which could therefore introduce a substantial source of error variance into the study (31). Interested potential subjects will be contacted by the study recruiter. If potential subjects agree, a 15 – 30 minute phone discussion will take place to determine whether they might be a match for the study. If the phone conversation indicates that the potential subjects may be a match for the study and they are still interested, they will be scheduled to meet with the study coordinator or another qualified study team member in person at the SFVAMC for informed consent and further eligibility procedures.

4.1 Subject Inclusion Criteria

Subjects must meet all inclusion criteria in order to be eligible for the study:

- 1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments;
- 2.) Habitual wake time between 0600 hr and 0800 hr maintained within the past month;
- 3.) Habitual bedtime between 2200 hr and 0000 hr maintained within the past month;
- 4.) Body Mass Index (BMI) >18 and < 28 kg/m²;
- 5.) Ability to communicate well with the Investigator and to understand the study requirements.

4.2 Subject Exclusion Criteria

Any of the following criteria will exclude the subject from entering the study:

- 1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI) (43);
- 2.) Current presence of two or more risk categories on the Berlin Questionnaire (42) for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea.
- 3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of depressive disorder

- not otherwise specified, assessed using the Structured Clinical Interview for DSM-IV TR Axis I Disorders (SCID) (41);
- 4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID (41);
 - 5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study;
 - 6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for ≥ 10 minutes which resulted in the development of persistent symptoms lasting ≥ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent.
 - 7.) Systemic illness affecting central nervous system (CNS) function;
 - 8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia;
 - 9.) Asthma or other reactive airway diseases;
 - 10.) Hepatic impairment (Child-Pugh A, B, C);
 - 11.) Any other chronic or unstable medical conditions;
 - 12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor;
 - 13.) Treatment with another investigational drug;
 - 14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor;
 - 15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization;
 - 16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization;
 - 17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day;
 - 18.) Self-reported consumption of alcohol within the past 30 days of > 14 standard drinks per week or ≥ 5 standard drinks on any day (men), or > 7 standard drinks per week or ≥ 4 standard drinks on any day (women).
 - 19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen;
 - 20.) Known liver disease or abnormal liver function tests assessed at the time of screening;
 - 21.) Self-reported regular caffeine use in excess of 200 mg per day on average within six months of screening;
 - 22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours);
 - 23.) Shift work within one month prior to the screening visit or planned shift work during the study;

- 24.) Travel of > 3 time zones within one week prior to the screening visit or any other visit;
- 25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.

5. STUDY DRUG HANDLING

5.1 Allocation to Dosing Groups

Subjects will be randomly assigned to one of four dosing groups in a 1:1:1:1 ratio: almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo. Randomization will be stratified based on gender and caffeine use. Subjects will dose one time on Study Day 10 at 1500 hrs according to their assigned dosing group.

Almorexant (100mg and 200mg) is currently being investigated in a comprehensive Phase III program. Results indicate that almorexant was well-tolerated in the initial Phase III study. Further Phase III studies to evaluate long-term efficacy and safety are in preparation (4).

Zolpidem 10mg is an imidazopyridine class sedative hypnotic which received original United States market approval under the brand name Ambien® in 1992.

5.2 Breaking the Blind

The blind will be maintained through study completion except for cases of breaking the blind due to emergency medical necessity. In situations in which the CCRC nursing staff or other study personnel determines that it might be necessary to break the blind, he/she will be instructed to contact the Investigator-Sponsor or Medical Monitor. If approval is granted by the Investigator-Sponsor or Medical Monitor, the CCRC nurse will be authorized to contact the research pharmacist at the CCRC. The research pharmacist will maintain a master randomization list and he/she or an authorized designee will be available to break the blind if necessary.

5.3 Dosing Adherence/Study Compliance

Since only one dose will be administered to subjects by a nurse at the CCRC, deviations from the scheduled dosing regimen are not anticipated.

During the sleep-wake monitoring period which will take place throughout the week prior to admission to the CCRC, subjects will be required to maintain regular wake times between 0600 hr and 0800 hr and bedtimes between 2200 hr and 0000 hr. Additionally, subjects will be asked to avoid recreational drug use, naps, the consumption of grapefruit or grapefruit juice, alcohol, and/or nicotine. Subjects will also be asked to maintain stable caffeine use and to avoid crossing more than three time zones. Actigraphs will be utilized to monitor the subjects' sleep-wake patterns and will therefore serve as a check for compliance with the prescribed sleep regimen. Subjects will maintain daily sleep diaries

during the 10-day study period which will capture the following items: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating of sleep quality on a scale of 1-100, caffeine use, and atypical events. Actigraphy and sleep diary data will be reviewed upon admission to the CCRC to determine compliance with the required sleep/wake schedule. An additional urine toxicology screening will be administered at the time of admission to the CCRC to rule out recent recreational drug use, and females will receive a urine pregnancy test at this time.

5.4 Drug Supplies

5.4.1 Formulation and Packaging

Actelion Pharmaceuticals Ltd. will provide almorexant 100 mg tablets, zolpidem 10 mg capsules, and matching placebo tablets and capsules. A double dummy design will be employed which will result in each subject receiving two tablets and one capsule. Study drug will be provided in bulk and will be shipped directly to the research pharmacy at the CCRC.

5.4.2 Preparing and Dispensing

The research pharmacist in the CCRC will maintain a copy of the randomization schedule and will receive the subject's randomization assignment at the time of hospital admission. The research pharmacist will dispense the assigned study drug to the nurse who will be administering the drug to the randomized subject.

5.4.3 Drug Administration

After obtaining the appropriate study drug from the research pharmacy, a CCRC nurse will administer the drug to the subject.

5.5 Drug Storage and Accountability

All drug products will be stored at the recommended temperature (room temperature at a maximum of 25°C). Site personnel and study monitors will perform regular checks to document that the study drug is stored appropriately and is within the defined expiration period at all times. A drug accountability log will be completed by the research pharmacist when study drug is received and dispensed to subjects. Any unused drug will be destroyed at the conclusion of the study.

5.6 Concomitant Medications

Medication use will be assessed at screening. Concomitant medications will also be assessed when the subject arrives at the CCRC on Day 8, on each subsequent day in the CCRC (Days 9, 10, and 11), and at follow-up. All concomitant medications will be recorded in the source documents and transcribed onto the Case Report Forms (CRFs).

5.6.1 Disallowed Concomitant Medications and Dietary Restrictions

Use of statins, prescription or over-the-counter medications containing psychoactive properties or stimulants is exclusionary and is also prohibited during the study period. Subjects will be required to maintain stable caffeine consumption of 200 mg per day or less during the study. Alcohol, recreational drug, and nicotine use is prohibited during the 10 day study period. Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization is prohibited.

6. STUDY PROCEDURES

6.1 Pre-Dosing Procedures

Screening (Days – 13 to 0)

The study coordinator or another qualified, trained study team member will obtain informed consent from each potential subject prior to the initiation of eligibility procedures. During the informed consent meeting, the study will be explained and the subject's questions will be answered. Subjects will be allowed to take as much time as they need to make a decision and will be given the option of discussing their decision with their family, friends, or other healthcare providers.

- Physical Exam, Medical History, and Prior/Concomitant Medications Assessment (performed by a nurse practitioner at the SFDVAMC CRC).
- Laboratory Analysis of Blood and Urine Samples: A urine sample and approximately 20ccs of blood will be collected for laboratory tests which will include a serum chemistry panel, liver function tests (including albumin), thyroid function tests, prothrombin time, complete blood count and differential, urine toxicology screen, and a urine pregnancy test (in women of childbearing potential). If lab values are out of range, subjects may be asked to repeat the blood draw for a retest to confirm their medical health.
- Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (SCID [41]), performed by a trained mental health clinician
- Self-report Berlin Questionnaire (42) to determine likelihood of sleep disordered breathing. If subjects have two or more positive scoring categories, they will also be monitored with pulse oximetry.
- Self-report Pittsburgh Sleep Quality Index (PSQI [43])
- Review of Inclusion/Exclusion Criteria

All screening assessments will be performed at the SFDVAMC, including the collection of blood and urine samples and laboratory analysis.

Sleep/Wake Monitoring (Days 1 to 7)

A seven-day sleep/wake baseline monitoring period will be scheduled for subjects who meet all inclusion and exclusion criteria. For female subjects, the baseline monitoring period will be scheduled such that Days 8 - 10 correspond to the follicular phase of the menstrual cycle. Prior to the start of the baseline week, a practice version of the PVT will be administered. Additionally, the Vocabulary Subtest of the Wechsler Adult Intelligence Scale, Fourth Edition (WAIS-IV [48]) will be administered for the purpose of obtaining an IQ measure to ensure that all dosing groups are matched on intelligence. Subjects will be asked to wear wrist actigraphs 24 hours per day on each day of the seven day monitoring period, and they will also be asked to abide by the following instructions:

- Adhere to a consistent wake schedule of 0600 hrs – 0800 hrs and a lights out schedule of 2200 hrs – 0000 hrs.
- Avoid nicotine and recreational drug use.
- Maintain stable caffeine consumption of ≤ 200 mg per day.
- Avoid alcohol use.
- Avoid the consumption of grapefruit or grapefruit juice.
- Avoid travelling > 3 time zones.
- Avoid naps.
- Avoid starting new medications unless they become necessary in the opinion of a physician.
- Use an acceptable form of birth control.

Subjects will maintain daily sleep diaries during the sleep/wake monitoring period which will capture the following data points: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating of sleep quality on a scale of 1 - 100, caffeine use, and atypical events.

Day 8 (CCRC Admission)

Subjects will enter the CCRC in the evening and a urine toxicology screen will be performed. A urine pregnancy test will be performed for female subjects of childbearing potential. Whether or not female subjects are in the follicular phase of their menstrual cycles will also be assessed at the time of admission. All subjects will be asked to report concomitant medications and AEs dating back to informed consent. Actigraphs will be collected and sleep diary data will be reviewed to determine compliance with the required sleep/wake regimen. Compliance with other study-related instructions will also be assessed at this time. While at the CCRC, subjects will receive a prescribed lights out time which will be consistent with the lights out regimen that was followed during the baseline week. All subjects will be prescribed a 0700hr wake time during their stay at the CCRC.

Subjects will continue to maintain a daily sleep diary during their stay at the CCRC. Additionally, during each night at the CCRC, subjects will have their sleep monitored with ambulatory PSG. Subjects will also be screened for obstructive sleep apnea which will involve thermistor measurements, pulse oximetry for detection of oxygen desaturation events, and two channels of respiratory inductive plethysmography to

measure chest and abdominal movement during breathing. Subjects with an apnea/hypopnea index ≥ 10 will be excluded from the data analysis.

Day 9

Subjects will be awakened at 0700 hrs and will remain in the CCRC for monitoring. Caffeine consumption should remain consistent with what the subject consumed throughout the sleep/wake monitoring period. Naps will be prohibited. During the evening (prior to lights out), AEs and concomitant medications will be assessed. Subjects will have their sleep monitored with PSG.

Day 10 (Pre-Dose; 0700hrs – 1500hrs)

Subjects will be awakened at 0700 hrs. Caffeine consumption will remain consistent with what the subject consumed throughout the sleep/wake monitoring period. Beginning at 1000 hrs, subjects will be administered a series of baseline (pre-dose) neurocognitive assessments, objective alertness assessments, and subjective assessments. All assessments will be administered by qualified, trained, research technicians. Assessments to be administered are described below:

Stanford Sleepiness Scale: Subjects will be asked to rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy). This scale will be administered just prior to each administration of the MWT. Administration time is less than 5 minutes.

Maintenance of Wakefulness Test: Subjects will be placed in a dimly lit room where they will sit comfortably and receive instruction to keep their eyes open and attempt to remain awake while being monitored via standard MWT EEG leads. If the subject falls asleep, he/she will be awakened after three epochs of sleep as determined by EEG trace. Administration time is 20 minutes.

Psychomotor Vigilance Test: Subjects will be required to press a button each time a target is presented. Administration time is 10 minutes.

Rey Auditory Verbal Learning Test – List 1: Each subject will be read a list of 15 words and asked to repeat back as many as they can remember. The task is repeated 4 more times. Subsequently, a new interference list is read and the subject is asked to repeat back items from that list. Then the subject is asked to recall items from the original list. Administration time is approximately 10 minutes.

Continuous Performance Test II: Subjects will be required to press the space bar or click the mouse button when any letter except for the target letter “X” appears. Administration time is 15 minutes.

Symptom Checklist: Subjects will be asked if they are experiencing specific symptoms commonly associated with hypnotics. If they endorse any of the symptoms on the

checklist, they will be asked whether the symptoms are mild, moderate, or severe. Administration time is approximately 5 minutes.

Vital signs (sitting blood pressure and heart rate) will be obtained several times throughout Day 10. Staff will also query for AEs at these time points.

6.2 Study Dosing

Day 10 (Dosing and Post-Dose, 1500hrs - 2200hrs)

Subjects will dose at 1500 hrs. Shortly after dosing, a PVT administration will take place. MWTs (preceded by the SSS each time) will be administered at 1530 hrs, 1730 hrs, 1930 hrs, and 2130 hrs.

Based on the literature (3), it is estimated that almorexant will reach peak blood concentration between 1600 hrs and 1800 hrs. Around this timeframe, subjects will be administered the PVT, CPT, and SC, in addition to the MWT and SSS. The following neurocognitive assessments will also be administered during this timeframe:

Paired-Associates Learning Task: Subjects will be read 10 pairs of words. They will then be read, in a different order, the first word from each pair for which they are to recall the associated second word. The list will be presented and followed by recall two more times (with pairs in a different order each time). The first administration of the Paired-Associates Learning Task (given during the timeframe of 1600hrs – 1800hrs) will test immediate recall, during which errors are corrected. The test will be administered again several hours after the first administration using the same word list to assess delayed recall. Errors will not be corrected during the delayed recall trial.

Rey Auditory Verbal Learning Test – List 2: The RAVLT will be administered again during the 1600 – 1800hrs timeframe, but with a new list.

Grooved Pegboard Test: The test consists of 25 holes with randomly positioned slots. Pegs with a key along one side must be rotated to match the hole before they can be inserted and subjects must place the pegs in the holes as quickly as possible. Administration time is approximately 10 minutes.

Stroop Color-Word Test: Subjects will be given three sheets of paper, one at a time. The Word page consists of the words “red,” “green,” and “blue” printed randomly in rows in black ink. Subjects will be asked to read as many words as they can out loud in a 45 second time period. The Color page consists of 100 items, all written as “XXXX,” printed in either green, red, or blue ink. Subjects will be asked to name as many colors as they can out loud in a 45 second time period. The Color-Word page consists of the words from the Word page printed in the colors from the Color page. The words and the colors they are printed in do not match one another. Subjects will be asked to name as many colors as they can in a 45 second time period. Total administration time is approximately 10 minutes.

Tower Test from Delis-Kaplan Executive Function System: Subjects will be asked to complete problem-solving tasks which will involve moving disks on pegs to match an arrangement shown to them in a picture. Administration time is approximately 20 minutes.

Digit Span: Subjects will be read a sequence of digits and asked to repeat the digits in the same sequence. For the second portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in reverse order. For the third portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in order from the lowest number to the highest. Administration time is approximately 6 minutes.

After the time window of 1600 hrs - 1800 hrs, subjects will receive additional administrations of the PVT, SC, and RAVLT (third list). Two more MWT administrations will also take place. The final assessment will begin at 2130 hrs.

Study personnel will remain with the subjects throughout testing and subjects will be kept awake until all assessments have been completed. Some of the neurocognitive tests will be audio recorded for quality control purposes.

Night 10 (Post-Dose)

AEs will be assessed prior to the prescribed lights out time. Subjects will engage in undisturbed, PSG recorded sleep.

Day 11 (Discharge)

Upon awakening at 0700 hrs, subjects will have all electrodes removed and will be debriefed prior to being discharged from the CCRC. AEs will be assessed prior to discharge.

Safety Follow-Up

Within 5 – 12 days of dosing with study drug, subjects will be required to have a blood draw performed for a liver function test. This procedure will be performed at the SFDVAMC. Approximately 5ccs of blood will be drawn and analyzed at the SFDVAMC laboratory. If lab values are out of range, the subject may be asked to repeat the blood draw for a retest. The occurrence of AEs and concomitant medications since the day of discharge will be assessed.

7. STUDY OUTCOMES AND SAFETY ASSESSMENTS

7.1 Study Outcome Assessment Measures

A description of the measures which will be utilized for the outcome analyses is provided below:

Psychomotor Vigilance Test: The PVT is a widely used instrument that measures sustained attention and reaction time (49). Extensive work with this measure has demonstrated that the PVT is not affected by practice effects and is a highly sensitive measure of the effects of disrupted circadian rhythms from shift work (17) and chronic sleep deprivation (18, 19). PVT-192® devices will be utilized for this study. The PVT has a random inter-stimulus interval of 2-10 seconds and can be collected over a 10 minute period. The main measure will be performance lapses (reaction time > 500 ms) per 10 minute period. Secondary measures will include total time of lapses, frequency of false responses, frequency of non-responses, durations of the 10% fastest and 10% slowest responses, and performance decrement across time on the task.

Stanford Sleepiness Scale: The SSS is a subjective measure of sleepiness in which subjects rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy) (50). Subjective sleepiness ratings will be collected in order to verify the sedative effects of zolpidem and the two doses of almorexant.

Maintenance of Wakefulness Test: The MWT is widely used to demonstrate significant pre and post treatment differences in excessive sleepiness. Sleep onset is defined as the first occurrence of > 15 seconds of cumulative sleep in a 30 second epoch. Latency to the first 30 seconds of sleep will be scored online by the attending sleep technologist. The subject will be awakened within 90 seconds of falling asleep.

Rey Auditory Verbal Learning Test: The RAVLT is a word learning task and a measure of short-term auditory memory and learning, as well as delayed auditory memory (52, 53).

Grooved Pegboard Test: A measure of manipulative dexterity, this test requires complex visual-motor coordination (51).

Paired-Associates Learning Task: This associative learning sub-test of the Wechsler Memory Scale tests the ability to learn and recall pairs of words, some of which are related (e.g., north/south) and others which are unrelated (e.g., eagle/jury) (47). Immediate and delayed recall trials will be scored for the number of correctly recalled pairs.

Continuous Performance Test II: The CPT assesses attention and working memory as well as executive function (44). Specifically, the CPT measures response inhibition via commissions (an aspect of executive function) and sustained attention via omissions. There is evidence in the literature which suggests that continuous performance tasks are sensitive to sleep-inducing agents (34). Scores will be based on response time and errors, inclusive of omissions and commissions.

Stroop Color-Word Test: The Stroop is a widely used putative measure of executive function that measures response inhibition (35). The Color-Word score will be computed,

which measures the subject's ability to inhibit or override the tendency to produce the more automatic or dominant response (i.e., to name the color word rather than the color).

Tower Test from Delis-Kaplan Executive Function System: D-KEFS Tower is typically used for the assessment of executive function, specifically to detect deficits in planning, decision making, and problem solving (45). Literature provides evidence of a link between performance on towers tasks and sleep (32).

Digit Span: Digit Span is a subtest of the WAIS-IV which measures attention and working memory and has been found to be sensitive to sleep-inducing agents (36, 48).

The following measures will serve as covariates:

Actigraphy: The primary actigraph measures are habitual sleep onset and offset times and the range of variability around these data points. The wrist actigraph provides continuous activity data using a battery-operated wristwatch-sized microprocessor that senses motion with an accelerometer. Subjects can also indicate lights on, lights off, and other salient events by pressing an event marker on the actigraphs. The actigraphs will be initialized with the ActMe program (Ambulatory Monitoring, Inc.) using the PIM sampling mode in one-minute epochs for conventional actigraphic sleep-wake estimation.

Polysomnography: The primary PSG measure is total sleep time on the night prior to the day of dosing and neurocognitive testing. PSG recordings will be obtained with ambulatory PSG and the parameters recorded will follow current guidelines as defined in the AASM Manual for the Scoring of Sleep and Associated Events (37).

The Embla Titanium ambulatory recorders record up to 34 channels. The sampling frequency ranges from 256Hz to 512 Hz. High and low frequency filters will be added while scoring the data manually and in spectral analysis. 60Hz notch filters may be applied to remove electrical noise. Raw files will be kept with only anti-aliasing filters. Spectral analysis will organize sleep epochs by stage and time. Artifacts will be tagged for removal for spectral analysis.

7.2 Safety Assessment Measures

Symptom Checklist: This checklist captures common symptoms experienced by subjects taking hypnotic medications. Reports of symptoms will be collected in order to compare possible drug side effects.

AEs will be assessed on a regular basis throughout the study and at the follow-up visit.

A liver function test will be performed on all subjects within 5 – 12 days of dosing with study drug.

8. ADVERSE EVENT REPORTING

8.1 Adverse Event Definitions

An AE is defined as any untoward medical occurrence that takes place in a clinical study, regardless of the causal relationship of the event with the investigational drug or study treatment(s). Any event occurring after the clinical trial participant has signed the study informed consent documentation should be recorded and reported as an AE.

An AE can be any unfavorable and unintended sign, symptom, or disease temporally associated with the use of the investigational product, whether or not considered related to the investigational product. A new condition or the worsening of a pre-existing condition will be considered an AE.

An abnormal test finding will be classified as an AE if one or more of the following criteria are met: a.) the test finding is accompanied by clinical symptoms; b.) the test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention, including significant additional concomitant drug or other therapy; c.) the test finding leads to discontinuation of subject participation in the clinical study; d.) the test finding is considered an AE by the Investigator-Sponsor of the IND application.

For each AE, the date and time of onset, a description of the event, severity, seriousness, action taken, relationship to the study drug, outcome, and date of resolution will be recorded.

A **Serious Adverse Event (SAE)** is defined as an AE that results in any of the following:

- Death
- Life-threatening event – An event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
- Requires hospitalization or prolongs existing inpatient hospitalization, not inclusive of a pre-planned elective hospital admission for treatment of a pre-existing condition that has not significantly worsened or a diagnostic procedure.
- Results in persistent or significant disability or incapacity.
- Results in congenital abnormality or birth defect.
- An important medical event occurs which requires medical intervention to prevent any of the above outcomes. Important medical events are those which may not be immediately life-threatening but may jeopardize the subject and may require intervention to prevent one of the serious outcomes listed above.

An **Unexpected Adverse Event** is defined as any AE in which the frequency, specificity, or severity is not consistent with the risk information described in the clinical protocol or elsewhere in the current IND application or Investigator's Brochure.

8.2 Recording Requirements

8.2.1 Eliciting Adverse Event Information

AEs will be assessed when subjects check into the CCRC and again during each evening at the CCRC. Additionally, subjects will complete a Symptom Checklist at various scheduled time points throughout the day of dosing and asked to report the occurrence of any other AEs.

8.2.2 Recording Requirements

All observed or volunteered adverse drug events (serious or non-serious) and abnormal test findings, regardless of treatment group or suspected causal relationship to the investigational drug or study treatment(s) will be recorded in the subjects' case histories. For all AEs, sufficient information will be pursued and/or obtained so as to permit a.) an adequate determination of the outcome of the event; and b.) an assessment of the causal relationship between the AE and the study drug.

AEs or abnormal test findings felt to be associated with the investigational drug or study treatment(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the Investigator-Sponsor.

8.3 Reporting of Adverse Events

8.3.1 Reporting of Adverse Events to the FDA

Written IND Safety Reports

The Investigator-Sponsor will submit a written IND Safety Report to the responsible new drug review division of the FDA for any observed or volunteered AE that is determined to be a.) associated with the investigational drug or study treatment(s); b.) serious; and c.) unexpected. Each IND Safety Report will be prominently labeled, "IND Safety Report."

Written IND Safety Reports will be submitted to the FDA as soon as possible and within 15 calendar days following the Investigator-Sponsor's receipt of the respective AE information. For each written IND Safety Report, the Investigator-Sponsor will identify all previously submitted IND Safety Reports that addressed a similar AE experience and will provide an analysis of the significance of newly reported AE in light of the previous, similar report(s).

Follow-up information to an IND Safety Report will be submitted to the applicable review division of the FDA as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require a written IND Safety Report does, in fact, meet the requirements for reporting; the Investigator-Sponsor will submit a written IND Safety Report as soon as possible and within 15 calendar days after the determination was made.

In accordance with FDA requirements, annual safety reports will be submitted to the FDA.

Telephoned IND Safety Reports

In addition to the subsequent submission of a written IND Safety Report (i.e., completed FDA Form 3500A), the Investigator-Sponsor will notify the responsible review division of the FDA by telephone or facsimile transmission of any observed or volunteered AE that is a.) associated with the use of the investigational drug or study treatment(s); b.) fatal or life-threatening; and c.) unexpected.

The telephone or facsimile transmission of applicable IND Safety Reports will be made as soon as possible but in no event later than 7 calendar days after the Investigator-Sponsor's initial receipt of the respective human AE information.

8.3.2 Reporting Adverse Events to the Responsible IRBs

In accordance with applicable IRB policies of the Veterans Affairs Medical Center Research and Development Committee, University of California, San Francisco Committee on Human Research, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO), the Investigator-Sponsor will report, to the IRBs, any observed or volunteered AE that is determined to be associated with the investigational drug or study treatment(s), serious, and unexpected. AE reports will be submitted to the IRBs in accordance with the respective IRB procedures.

Applicable AEs will be reported to the IRBs as soon as possible and, in no event, later than 10 calendar days following the Investigator-Sponsor's receipt of the respective information. Follow-up information to reported AEs will be submitted to the IRB as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting, the Investigator-Sponsor will report the AE to the IRB as soon as possible, but in no event later than 10 calendar days after the determination was made.

In accordance with the USAMRMC HRPO requirements, unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all subject deaths related to participation in the study should be promptly reported by phone (310-619-2165), by e-mail (hsrrb@amedd.army.mil), or by facsimile (301-619-7803) to the USAMRMC HRPO. A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the USAMRMC, ATTN: MCMR-ZB-P, 504 Scott Street, Fort Detrick, Maryland, 21702-5012.

The Medical Monitor is required to review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the

protocol and provide an unbiased written report of the event to the USAMRMC HRPO. At a minimum, the Medical Monitor should comment on the outcomes of the event or problem and in the case of a serious adverse event or death, comment on the relationship to participation in the study. The Medical Monitor should also indicate whether he/she concurs with the details of the report provided by the Investigator-Sponsor. Reports for events determined by either the Investigator-Sponsor or Medical Monitor to be possibly or definitely related to participation and reports of events resulting in death will be promptly forwarded to the HRPO.

8.3.3 Reporting of Adverse Events to Actelion Pharmaceuticals

Copies of all periodic safety reports (including draft versions for review) to be submitted to the FDA will be provided to Actelion at least 10 days prior to their submission to the FDA. Copies of any MedWatch forms submitted to the FDA will be provided to Actelion immediately upon submission to the FDA.

All serious adverse events, regardless of causality and expectedness, will be reported to Actelion within 24 hours of the Investigator-Sponsor's knowledge of the event.

8.3.4 Withdrawal of Subjects Due to Adverse Events

Withdrawal of subjects due to an AE can take place at any time during the study at the discretion of the Investigator-Sponsor. Subjects may also choose to discontinue participation at any time.

9. STATISTICAL METHODS/DATA ANALYSIS

9.1 Study Endpoints

9.1.1 Analysis of Primary Endpoints

It is hypothesized that subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance and objective measures of sleepiness compared to subjects receiving placebo, almorexant 100mg, or almorexant 200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. When multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (pre- or post-medication) interaction effect serving as the test of the hypothesis. When a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Covariates in all models will include total sleep time measured by PSG on the night before testing and average sleep duration measured by actigraphy. Planned comparisons will be conducted to compare the zolpidem 10mg group with placebo, almorexant 100mg, and almorexant 200mg separately. Post-hoc comparisons will be made to compare placebo vs. almorexant 100mg, placebo vs. almorexant 200mg, and almorexant 100mg vs. almorexant 200mg. For post-hoc

comparisons, p-value adjustments will be made using a re-sampling procedure as implemented in the SAS “simulate” adjustment option.

Two-tailed significance tests will be conducted at the $p = .05$ level. P-value adjustments will be made for multiple endpoint variables within each domain of neurocognitive functioning (verbal memory, attention/working memory, motor skills, executive function, and psychomotor vigilance) and objective sleepiness (sleep onset latency and low frequency EEG power in the MWT). The p-value adjustments will be made using a step-down, re-sampling based procedure (38, 39) which takes into account the correlational structure among the multiple variables. Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. Dropout rate will itself be analyzed as a secondary outcome variable. Missing data will be carefully characterized, and multiple imputation will be used where necessary. The exact form of each mixed model, for example the correlational structure of repeated measures and whether heterogeneous group variances are included, will be made on the basis of best fit according to the Bayesian Information Criterion (BIC) before any hypothesis testing is conducted. Assumptions of the models (e.g., normal distributions of errors and absence of outliers) will be assessed, and any necessary remedies, such as data transformation or the use of robust standard errors, will be implemented before hypothesis tests are conducted.

Any deviations from the statistical plan will be described in the study manuscript.

9.1.2 Analysis of Secondary Endpoints

Secondary endpoints include sleep latency on the MWT measured beyond the presumed drug activity period at 270 and 390 minutes post-dose (i.e., the “hangover effect”), and subjective sleepiness measured by the Stanford Sleepiness Scale. Secondary analyses will be conducted in a parallel fashion to the primary analyses, but with re-sampling based multiple comparison procedures for all significance tests.

9.2 Sample Size Determination

Enrollment is estimated to include up to 216 subjects to obtain 200 evaluable subjects. An equal number of subjects (up to 54) will be randomly assigned to each dosing group (almorexant 100 mg, almorexant 200 mg, zolpidem 10 mg, placebo). Randomization will be stratified on the basis of gender and caffeine use. With a power of 0.80 and an alpha of 0.05, the planned sample size will allow for the detection of effect sizes (Cohens' f) of approximately 0.29. It is estimated that the effect of zolpidem 10 mg versus placebo on the cognitive performance measures will range from $f = 0.34$ to $f = 0.80$, based on prior findings. Given the hypothesis that both doses of almorexant will be associated with significantly less impairment than zolpidem 10mg, it is possible that a range of effect sizes might be found with almorexant. If almorexant is absolutely no different than placebo, the study will be slightly overpowered to demonstrate its superiority over zolpidem. However, if almorexant has a more subtle impairment effect on cognition, intermediate between that seen with zolpidem 10 mg and placebo, it might become

necessary to be able to detect somewhat smaller effects. According to guidelines suggested by Cohen (33), an effect size of $f = .14$ is considered “small” and $f = .39$ is considered “medium.” Thus, the proposed study is well powered to test its main hypotheses.

9.3 Definition of Analysis Populations

Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. If there are a substantial number of participant dropouts, separate analyses on completers only will be conducted as a sensitivity analysis, but hypothesis tests will be based on the intent-to-treat sample. No subgroup analyses are planned.

9.4 Safety Analysis

Dosing groups will be compared on each symptom included as part of the Symptom Checklist using Fisher’s exact tests or Chi-Square approximations, depending on the frequency of each symptom. No p-value adjustments will be made.

10. QUALITY CONTROL (QC) AND QUALITY ASSURANCE

The study will be carried out according to requirements of the FDA and all other applicable agencies in addition to ICH accepted standards of GCP. All study-specific procedures will be performed according to approved written Standard Operating Procedures. Study monitors will be responsible for ensuring adherence to FDA and ICH guidelines. Study Monitors for this study will be provided by an external contract monitoring group. Regular monitoring of study data and files at the clinical study sites will be performed as defined in the study-specific monitoring plan. Additionally, an authorized representative from the Investigator-Sponsor study team will perform an annual review of study files and training files to ensure adherence to GCP guidelines and study-specific standard operating procedures. Data collected during the study will be subjected to a thorough quality control review by the lead data managers prior to the statistical analysis. Specific requirements related to the data management QC of the study data will be detailed in the Data Management Plan. AE data will be reviewed on an ongoing basis with the Investigator-Sponsor.

11. DATA HANDLING, RECORD KEEPING, AND CONFIDENTIALITY

11.1 Data Recording/Case Report Forms (CRFs)

A CRF will be completed for each subject enrolled into the clinical study. The Investigator-Sponsor will review each completed CRF book and will complete the Investigator Statement. Completion of the Investigator Statement CRF confirms the Investigator-Sponsor’s responsibility for ensuring that all data and corrections on the CRF are complete, accurate, and authentic.

Source documents will consist of laboratory and medical history records, screening instruments, actigraphy data, sleep diaries, PSG data, neurocognitive assessments, and subjective symptom measures including the Symptom Checklist, the Stanford Sleepiness Scale, and AE and concomitant medication disclosures. All necessary information from the source documents will be recorded on the CRFs. Where appropriate, certain data files will be merged with the study database electronically. Data recorded on the CRFs will be identical to the data recorded on the source documents. Queries will be issued to address all discrepancies noted within the study data. Any changes made to the study data as the result of a resolved query will be documented in the audit trail within the study database. Specific procedures related to the handling of blank, discrepant, or otherwise spurious data will be detailed in the Data Management Plan. When all data have been entered, validated and queries resolved, the database will be locked.

11.2 Record Maintenance and Retention

The Investigator-Sponsor will maintain records in accordance with GCP guidelines and all applicable regulations and policies, to include:

- FDA correspondence related to the IND and clinical protocol, including copies of submitted Safety Reports and Annual Reports
- IRB correspondence (including approval notifications) related to the clinical protocol, including copies of AE reports and annual or interim reports
- Current and past versions of the IRB-approved clinical protocol and corresponding IRB-approved consent form(s) and, if applicable, subject recruitment advertisements
- Signed FDA Form 1572 Statements of Investigator
- Financial disclosure information
- Curriculum vitae for the Investigator-Sponsor and all clinical protocol sub-investigators and study personnel
- Certificates of required training for Investigator-Sponsor, all sub-investigators, and other relevant study team members
- Listing of printed names/signatures of Investigator-Sponsor and listed sub-investigators
- Normal values for laboratory ranges
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational drug, other study treatments, and study materials
- Standard procedures for decoding and breaking the study blind
- Master randomization list
- Signed informed consent forms
- Completed Case Report Forms, signed and dated by the Investigator-Sponsor
- Source Documents
- Monitoring visit reports
- Copies of Investigator-Sponsor correspondence to sub-investigators, including notifications of safety information

- Subject screening and enrollment logs (a listing of all volunteers who signed informed consent)
- Subject identification code list
- Investigational drug dispensing and accountability records, including documentation of drug disposal
- Final clinical study report

The Investigator-Sponsor will retain the specified records and reports for a minimum of two years after the marketing application is approved for the investigational drug. If a marketing application is not submitted or approved for the investigational drug, records will be retained until 2 years after investigations under the IND have been discontinued and the FDA so notified.

11.3 Confidentiality

Participation in research will involve a loss of privacy, but information about subjects will be handled as confidentially as possible. Medical records will be created at UCSF and SFVAMC because of subjects' participation in this study. Information related to informed consent and screening test results will be included in the medical records, as well as information pertaining to vital signs, adverse events, and concomitant medications assessed during the hospital portion of the study. Therefore, other doctors may become aware of the individual's study participation. Hospital regulations require that all health care providers treat information in medical records confidentially. At the time of consent, subjects will be asked to sign forms to authorize the release of their personal health information for research purposes.

If it is suspected that the subject is in danger of harming him/herself or someone else, or if child abuse or neglect or elder abuse has occurred, appropriate authorities will be notified as required by law. It is also possible that subjects' research records could be subpoenaed by a court.

If information from this study is published or presented at scientific meetings, subjects' names and other personal information will not be used.

All study data will all be coded with a code number unique to the study. Only study personnel, with the permission of the Investigator-Sponsor, will have access to the key with the name and ID codes. The subject identification code list will be stored electronically in a password-protected, restricted access folder on a secured study server in order to maintain confidentiality. The only individuals receiving access to the code list will be the team member responsible for maintaining the list and a back-up.

The clinical interviews performed at screening will be audio recorded and will be used only by research personnel in order to calibrate the clinicians' ratings on the standardized interview format. The recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encrypted, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion

of the study; at that point, they will be erased. Subjects will be informed that their screening clinical interviews will be audio recorded for the purpose of allowing the research team to ensure consistency across all clinical interviews. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

The Maintenance of Wakefulness Tests performed on Day 10 will be video recorded and will be used only by research personnel for the purpose of confirming subjects' ability to remain awake during the testing process. The recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encrypted, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion of the study; at that point, they will be erased. Subjects will be informed that their Maintenance of Wakefulness Tests on Day 10 will be video recorded for the purpose of allowing the research team to confirm their ability to remain awake during testing. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

Organizations that may look at and/or copy subjects' medical records for research, quality assurance, and data analysis include representatives from the following:

- UCSF CHR
- FDA
- USAMRMC
- Actelion Pharmaceuticals, Ltd.

12. ETHICS

12.1 Institutional Review Board (IRB) approval

Prior to initiating the study, the Investigator-Sponsor will obtain approval in writing from all required IRBs. Specifically, approval must be obtained from the UCSF Committee on Human Research, the Veterans Affairs Research and Development Committee, and the U.S. Army Medical Research and Materiel Command Office of Research Protections Human Research Protection Office.

Any amendments to the protocol or changes to the informed consent document must be approved by all IRBs prior to the implementation of those changes. The only circumstance in which a modification to the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator-Sponsor will promptly notify the IRBs of the modification.

The IRBs will be promptly notified of any deviation to the protocol that may have an effect on the safety of the subjects and the integrity of the study. This notification will occur as soon as the deviation is identified. All deviations will also be reported in the continuing review report and final study report.

A copy of the approved continuing review report and the local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available. A copy of the approved final study report and local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available.

In the event that the IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of an Investigator-Sponsor's decision to modify the previously accepted clinical protocol, the Investigator-Sponsor will submit a protocol amendment (prior to the implementation of the changes) to the IND describing any change to the protocol that would significantly affect the safety of subjects, the scope of the investigation, or the scientific quality of the study.

Records of IRB approval and other related correspondence will be maintained in the regulatory files for the study and will be subject to periodic audits and reviews by study monitors. Periodic status reports will be submitted to the IRB as required, and AEs/serious AEs will be reported to each IRB per their specific reporting requirements.

12.2 Ethical and Scientific Conduct of the Clinical Study

The clinical study will be conducted in accordance with the current IRB-approved clinical protocol, ICH Guidelines on GCP, and relevant policies, requirements, and regulations of the FDA, UCSF CHR, the VA R&D Committee, the USAMRMC ORP HRPO, and all other applicable state and federal agencies. All procedures described in this protocol will be performed according to approved written SOPs unless otherwise stated.

12.3 Subject Informed Consent

The Investigator-Sponsor will make certain that an appropriate informed consent process is in place to ensure that potential research subjects are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Investigator-Sponsor, or a staff member designated by the Investigator-Sponsor, will obtain the written, signed informed consent of each subject prior to performing any study-specific procedures. The date and time that the subject signs the informed consent form and a narrative of the issues discussed during the informed consent process will be documented in the subject's case history. The Investigator-Sponsor will retain the original copy of the signed informed consent form and a copy will be provided to the subject.

The Investigator-Sponsor will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Investigator-Sponsor will obtain the informed consent of enrolled subjects for continued participation in the clinical study

13. EARLY DISCONTINUATION CRITERIA

A subject may withdraw or be withdrawn from the study for the following reasons:

- 1.) Subject withdrew consent
- 2.) Investigator judgment
- 3.) Protocol violation(s)
- 4.) Non-compliance
- 5.) Adverse Event
- 6.) Pregnancy
- 7.) Other

If subjects withdraw consent prior to admission to the CCRC, they will be asked to return to the SFDVAMC for an early discontinuation visit which will entail an assessment of AEs and concomitant medications, a debriefing, and the return of study-related equipment.

If it becomes necessary to stop parts or all of the clinical study for the safety of the subjects, Actelion, the IRBs, and the FDA will be notified promptly of the discontinuation of the entire clinical study. Respective protocol modifications will be submitted prospectively to the IRB and to the FDA for discontinuation of parts of the clinical study. All sub-investigators will be notified of any necessary discontinuations.

Subjects participating in the study at the time of the discontinuation of a portion or all of the study will be promptly notified and advised of the impact of the discontinuation upon their study schedules. If a portion of the study is discontinued, subjects will be provided with revised informed consent documentation which will need to be signed prior to their continued enrollment in the study.

14. RISKS AND BENEFITS

Study-related risks and associated measures to minimize the risks are listed below:

Study Drug Related Side Effects

Some subjects might experience side effects associated with the study drugs. The list of possible side effects presented below is based on side effects that have been observed in clinical trials involving Almorexant and Zolpidem. Participants in these clinical studies

took many different dosages of these drugs ranging from 1mg to 1000mg. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug. All subjects will have a liver function test performed within 5 – 14 days of dosing with study drug.

Risks and side effects related to taking Almorexant include those which are:

Likely (occurring in greater than 20% of people)

- Drowsiness

Less Likely (occurring in less than or equal to 20% of people)

- Fatigue
- Headache
- Dizziness
- Nausea
- Liver Enzyme Elevations (mainly with administration for longer than two weeks of daily almorexant 100mg and 200mg)

Rare but Serious

- Heart rate abnormality (less than 1%)
- Convulsions (less than 1%)

Risks and side effects related to taking Zolpidem include those which are:

Less Likely (occurring in less than or equal to 20% of people)

- Dizziness
- Drowsiness
- Headache
- Diarrhea
- Fatigue

Rare but Serious

- Heart rate abnormality (less than 1%)
- Severe allergic reaction (less than 1%)

Blood Drawing (Venipuncture)

The risks of drawing blood include temporary discomfort from the needle stick, bruising, and rarely, infection. The amount of blood collected to determine eligibility is approximately 20 ccs or 4 teaspoons. Only a qualified phlebotomist will draw blood following standard SFVAMC lab procedures.

Clinical Interview & Questionnaires

The interview and questionnaires may be distressing to some participants. Subjects will be told that they are free to decline to answer any questions or to stop the interviews at any time. The interviewer will be available to immediately assist with any problems that arise in the interview and will make a referral if required.

Audio Recording – Clinical Interview and Neurocognitive Tests

The clinical interviews and some of the neurocognitive tests will be audio taped. The audio taping may make subjects somewhat more uncomfortable than they would be without the taping. Research personnel will use the recordings in order to ensure that

study staff are administering and scoring the tests correctly and in the same way. The audio recordings will be maintained under secured conditions (i.e., the recordings will be encrypted, protected with a pass code, and stored and accessed via a secure server), identified only by a unique ID number, and retained until the conclusion of the study, at which point they will be erased/deleted.

Actigraphy

There is no risk of injury from wearing the actigraph. Subjects might find it annoying to have to wear the actigraph 24 hours per day during the baseline week. Subjects will be told that they can discuss any difficulties with this procedure with study personnel at any time. Subjects will also be able to decline to participate in this procedure at any time.

Polysomnography

There is no risk of injury from any of the recording devices, but subjects might experience slight discomfort from the attached electrodes and tape. High quality hypoallergenic materials will be used to minimize this risk.

Video Recording – Maintenance of Wakefulness

The Maintenance of Wakefulness Tests that will be conducted on Study Day 10 will be videotaped. The video recording may make subjects somewhat more uncomfortable than they would be without the taping. These recordings will only be reviewed by research staff and our consultants for the purpose of confirming subjects' ability to remain awake during the testing. The recordings will be identified by a unique ID number and will be stored under secure conditions (i.e., they will be encrypted, protected with a pass code and stored on a secure server). The recordings will be retained until the conclusion of the study, at which point they will be destroyed.

Maintenance of Wakefulness Tests

There is no risk of injury from taking this test, but subjects might find it annoying or difficult to remain awake while sitting quietly in a comfortable position. Subjects might also become bored while sitting still for the 20 minute duration of the test. Subjects will be able to stop the procedure at any time if they become uncomfortable.

Neurocognitive Assessment Battery

There is no risk of injury from completing the neurocognitive assessment battery, but subjects might become bored, frustrated, or find it difficult to concentrate as you take these tests throughout the day of testing. Subjects will be able to stop the procedures at any time if they become uncomfortable.

Sleepiness

There is a 3 out of 4 chance that subjects will take a sleep aid on Study Day 10 while at the hospital. Therefore, subjects might become sleepy during the study testing procedures, and the study staff will require subjects to remain awake. This might be difficult or frustrating for subjects.

Reproductive Risks

Subjects should not become pregnant or father a baby while participating in this study because the potential effects of the study drugs on an unborn baby are not known at this time. Women should not breastfeed a baby while on this study. Study staff will educate subjects regarding the importance of using appropriate birth control throughout the study.

Unknown Risks

The experimental drugs used in this study may have side effects or discomforts that no one knows about yet. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug. Subjects will not experience any direct benefits by participating in the study. However, the study is contributing to medical knowledge related to the cognitive effects of sleep aids. Results could have implications for personnel of the military and/or other professions who have an occupational risk of poor sleep.

15. STUDY PERSONNEL**15.1 Investigator-Sponsor**

The Investigator-Sponsor will assume overall scientific and administrative leadership for the study. He will be responsible for supervising the study team with regards to the recruitment, diagnostic assessment, and enrollment of subjects and the coordination of all study procedures.

The Investigator-Sponsor will have overall responsibility for the standardization of data collection, data quality control, data analysis, and interpretation. He will have overall responsibility for subject safety, rights, and welfare. He will be an active participant in the preparation of abstracts and manuscripts and will assure the dissemination of study findings in the professional and scientific communities.

15.2 Medical Monitor

The Medical Monitor may be asked to discuss research progress with the Investigator-Sponsor, consult on individual cases, or evaluate adverse event reports for the safety and protection of the subjects. The Medical Monitor shall promptly report discrepancies or problems to the IRB and the HRPO, and he will have the authority to stop a research study in progress, remove individual subjects from a study, and take whatever steps are necessary to protect the safety and well-being of research volunteers until the IRB can assess the Medical Monitor's report. At a minimum, the Medical Monitor will provide a written opinion regarding the relationship and outcome of any unanticipated problems related to participation, serious adverse events, and subject deaths.

15.3 Co-Investigators

The Co-Investigators assigned to this study will assist the research team in data collection, data analysis, quality control of study data, data interpretation, and the

preparation of reports. They will provide consultation and oversight to the mental health clinicians and will assist with the determination of eligibility.

15.4 Study Coordinator

The study coordinator will be responsible for the day-to-day activities of the study, including but not limited to the following: obtaining informed consent, subject scheduling, eligibility determination, ensuring the completion of safety reports in a timely manner, case report form completion, ensuring that study team members are properly trained on study procedures, providing oversight to the external study monitors, and providing oversight for data completion, cleaning, analysis, and interpretation. The study coordinator will consult with the project director as necessary for high-level study management and budget oversight.

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Neuropsychological Testing Battery

Testing Day Time	Test	Avg. Admin Time
1000 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
1030hrs	PVT*	11 min
1040 hrs - 1200hrs	Break	1 hr 20 min
1200 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
1230 - 1330hrs	Lunch	1 hr
1330 hrs	RAVLT-List 1*	10 min
	Continuous Performance Task	16 min
	Symptom Checklist	5 min
1400-1500	Break	1 hour
1500 - 1510hrs	Dosing	10 min
1510 hrs	PVT*	10 min
1520 - 1530hrs	Break	10 min
1530 hrs	Stanford Sleepiness and Maintenance of Wake	25 min
1555 hrs	RAVLT Delayed*	4 min
	Continuous Performance Task	16 min
	P-A *	7 minutes
	RAVLT - List 2*	10 min
	Symptom Checklist	1 min
	Stroop	5 min
	Grooved Pegboard	8 min
1645hrs	PVT*	15min
1700hrs	Towers	20 min
	Digit Span	6 min
1730hrs	Stanford Sleepiness and Maintenance of Wake	30 min
	Symptom Checklist	1 min
1805 - 1910 hrs	Dinner	65 min
1910 hrs	PVT*	10 min
1920 hrs - 1930 hrs	Break	10 min
1930 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
2000 hrs	P-A delayed *	5 min
2005 hrs	Symptom Checklist	5 min
2010 hrs	RAVLT - List 3*	10 min
2020hrs - 2105hrs	Break	45 min
2105 hrs	PVT*	10 min
2115 - 2130 hrs	Break	15 min
2130 hrs	Stanford Sleepiness and Maintenance of Wake	30 min

* RAVLT = Rey Auditory Verbal Learning Test

* PVT = Psychomotor Vigilance Test

* P-A = Paired Associates Subtest of the Wechsler Memory Scale

Appendix 2: Animal Studies Progress Report

Award Number:

USAMRAA Grant W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE
PERFORMANCE

PRINCIPAL INVESTIGATOR:

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REPORT DATE:

July 30, 2011

TYPE OF REPORT:

Progress Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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1. REPORT DATE (DD-MM-YYYY) 29-07-2011	2. REPORT TYPE Progress Report	3. DATES COVERED (From - To) 09/01/2010 – 08/31/2011
4. TITLE AND SUBTITLE EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE		5a. CONTRACT NUMBER W81XWH-09-2-0081
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Kilduff, Thomas S., Ph.D.		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) SRI International 333 Ravenswood Avenue Menlo Park, CA 94025-3493		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION / AVAILABILITY STATEMENT		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT <p>During Year 2, a new laboratory for behavioral performance assessment and microdialysis sampling was occupied and two new HPLCs were purchased and calibrated. Both the benzodiazepine receptor agonist zolpidem (ZOL) and the hypocretin (Hcr) receptor antagonist almorexant (ALM) induced sleep in rodents. However, ALM did not impair performance in a spatial reference memory test whereas ZOL did. Preliminary results indicate that the wake-active Hcr neurons could be activated in the presence of ALM but not in the presence of ZOL. In contrast, a sleep-active cortical neuron population was equally activated by ALM and ZOL. ALM caused a significant decrease in basal forebrain (BF) glutamate and concurrent increased BF GABA and adenosine during NREM/REM sleep compared to ZOL or VEH. These results are consistent with the hypothesis that the disfacilitation of wake-promoting systems by ALM results in less functional impairment than the general inhibition of neural activity produced by ZOL.</p>		
15. SUBJECT TERMS <p>Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis</p>		

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 20	19a. NAME OF RESPONSIBLE PEI Thomas S. Kilduff, Ph.
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include code) 650-859-5509

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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PROGRESS REPORT

“Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance”

USAMRAA Grant W81XWH-09-2-0080

DR080789P1

Year 2: 8/1/10 to 7/29/11

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces fewer functional impairments than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish if ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

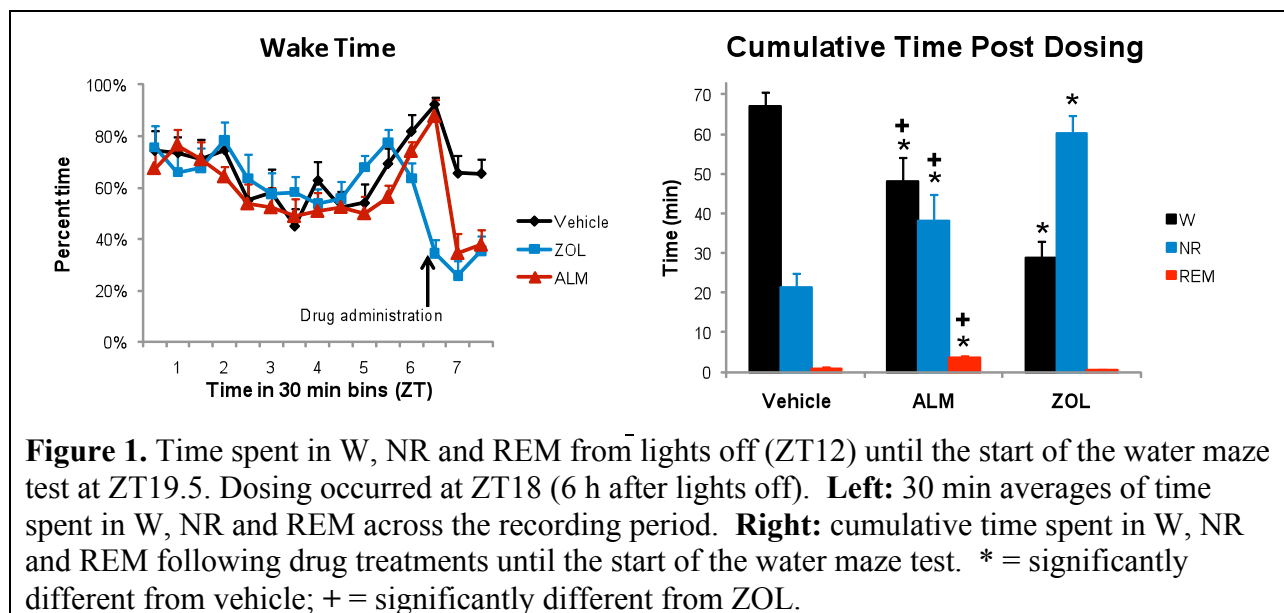
- 2a. Assessment of Almorexant effects on spatial reference memory in rats (months 1 to 12).
- 2b. Assessment of Almorexant effects on spatial working memory in rats (months 1 to 12).
- 2c. Assessment of Almorexant effects on psychomotor vigilance in rats (months 13 to 24).
- 2d. Synthesis of ALM (months 1-4). COMPLETED

Progress – Tasks 2a and 2b: Significant progress has been made on Task 2a. As discussed in last year’s progress report, the water maze (WM) and video tracking system was set up in temporary space while our permanent lab was under construction. One group of 24 rats was studied in the temporary space. Following completion of our permanent lab at the end of August, 2010, we moved the WM to room LW103A in this new facility. After extensive testing and revalidation of the experimental setup, all further experiments have been performed in LW103A.

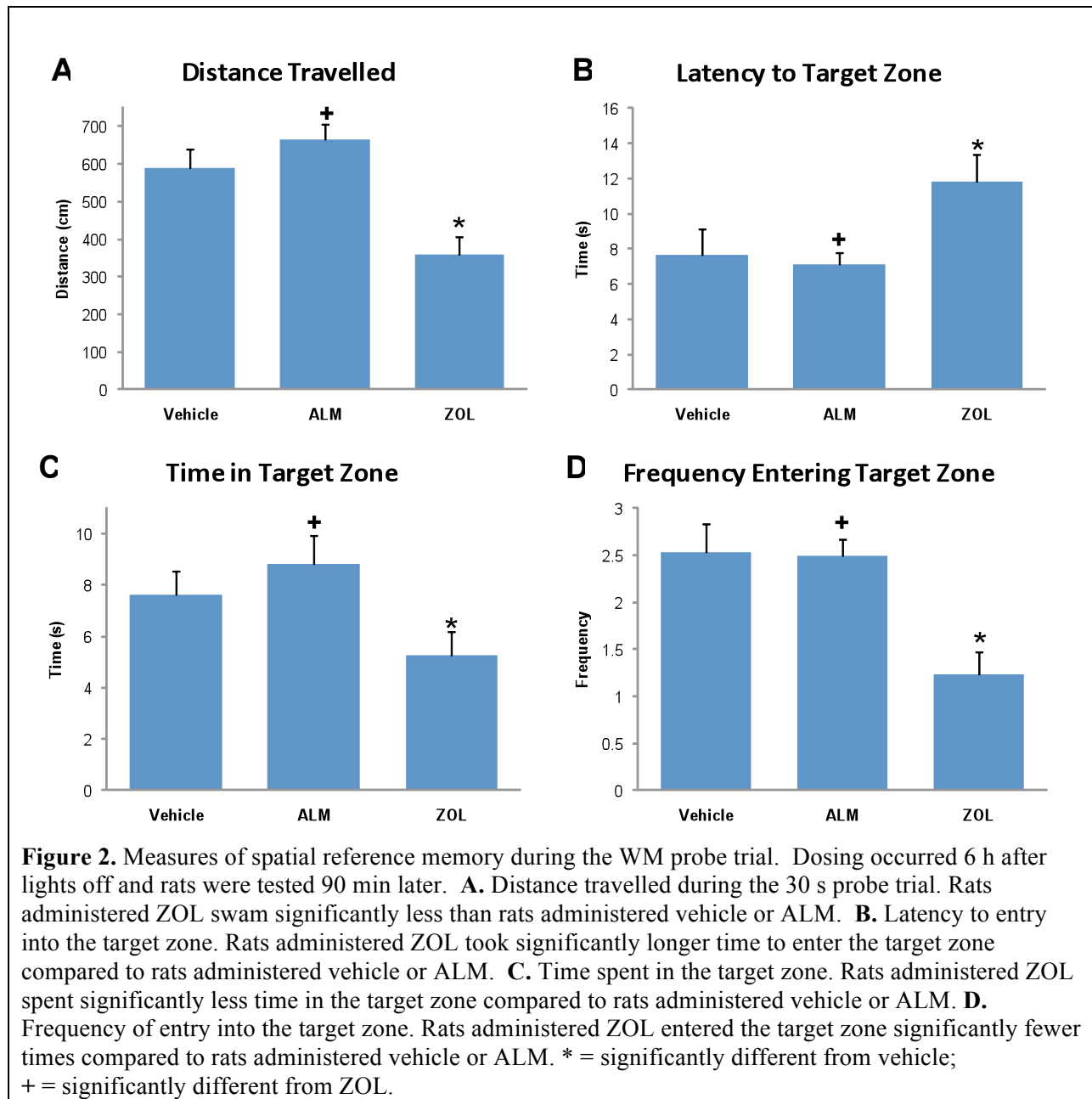
To date, 72 male Sprague-Dawley rats (325-350g) have been implanted with telemetry devices for recording electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_b) and locomotor activity (LMA). Following a minimum of 3 wks for recovery from surgery, each rat was recorded for a 24 h period to assess undisturbed sleep/wake patterns. The test for assessing the effects of Almorexant (ALM) on spatial reference memory in rats occurred on 2 consecutive days. On day 1, the acquisition of the task occurred in one session consisting of 8 consecutive WM trials with a 60 second (s) inter-trial interval. On the following day, rats were dosed 6 h into their active period (ZT18), left undisturbed for 90 min, and then a retention probe trial was performed. For this test, the platform was removed from the WM and the rats were allowed to swim and search for the platform for 30 s. Parameters measured during

the retention probe trial included the time and distance traveled in the quadrant of the WM where the platform had been on the acquisition day, as well as the latency and the number of entries into the target quadrant. EEG and EMG recordings were analyzed from the beginning of lights out (ZT12) until initiation of the WM test (7.5 h later). For more details on our experimental procedures, please see the full protocols in our original proposal.

Results: Both ALM (100 mg/kg i.p and p.o.) and zolpidem (ZOL, 30 mg/kg i.p. and 100 mg/kg p.o.) had significant sleep-promoting effects (Figure 1). Waking (W) was decreased and non-rapid eye movement sleep (NR) increased by ALM and ZOL compared to vehicle. Although NR was increased to a greater extent following ZOL than ALM, rapid eye movement sleep (REM) was increased significantly more by ALM compared to ZOL. Importantly, for the 30 min just prior to WM testing, both the ALM and ZOL groups of rats slept equivalent amounts. The differences between the sleep-promoting effects of ALM and ZOL occurred primarily during the first 30 min following drug administration. Confirming our previous findings, ZOL significantly reduced the latency to sleep onset compared to vehicle and ALM.



During the WM probe trial, rats administered ZOL showed impairments in all parameters measured compared to rats administered vehicle or ALM whereas ALM was indistinguishable from vehicle for all measures (Figure 2). Following ZOL, rats swam less, took longer to reach the target zone, spent less time in the target zone and entered the target zone less frequently compared to rats administered vehicle or ALM. These results support our initial hypothesis in which we predicted that rats would perform more poorly following ZOL than following ALM. These results demonstrate that, although ALM is a potent hypnotic, it impairs the performance of rats less than ZOL does in this task.



Task 3. Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.

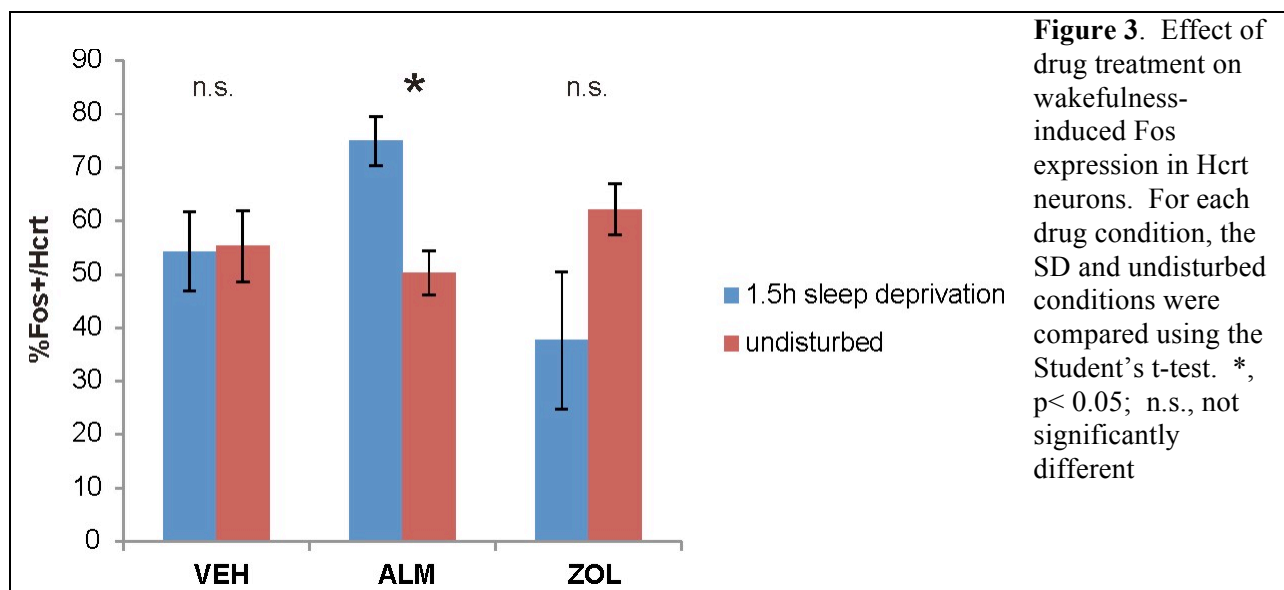
3a. Double-label immunohistochemistry with Fos and phenotypic markers (months 1 to 12).

3b. Assessment of hypnotic efficacy in saporin-lesioned rats (months 13 to 24).

3c. Assessment of hypnotic efficacy in transgenic mice (months 25 to 36).

Progress –Task 3a: To assess the influence of ALM and ZOL on the activity of sleep/wake regulatory neurons, we performed an immunohistological study using c-Fos as a marker of neuronal activity. Twenty of the rats prepared for Task 2 above were administered

ALM (100 mg/kg i.p.), ZOL (30 mg/kg i.p.), or vehicle at ZT18 as described above. Half of the animals in each drug treatment condition were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling. All animals were then deeply anesthetized, perfused and the brains sectioned. Double-label immunohistochemistry for the marker of functional activity, Fos, and the neuropeptide hypocretin (Hcrt; a “wake-active” hypothalamic neuronal population) was performed in coronal brain sections at the level of the lateral hypothalamus. Analysis of double-labeled neurons revealed that the wake-active Hcrt neurons showed higher levels of Fos expression after SD than after the undisturbed condition only in the ALM-treated rats (Fig. 3) whereas there was no such difference for the ZOL-treated rats. These preliminary results indicate that activation of the Hcrt neurons by SD is unimpaired in the presence of ALM whereas ZOL inhibits this population irrespective of behavioral state. A second batch of tissues from another cohort is currently being processed to increase the sample size for this experiment.



Task 4. Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.

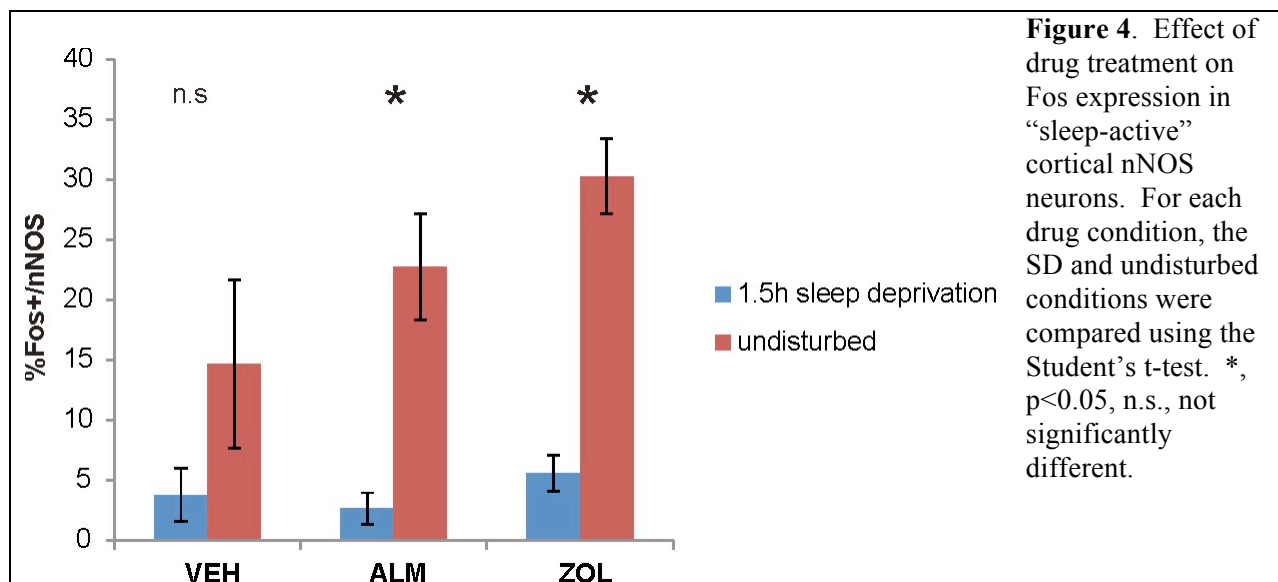
4a. Effects of ALM and ZOL on sleep-active brain areas (months 1 to 12).

4b. BF adenosine (ADO) release in response to oral ALM and ZOL (months 1 to 24).

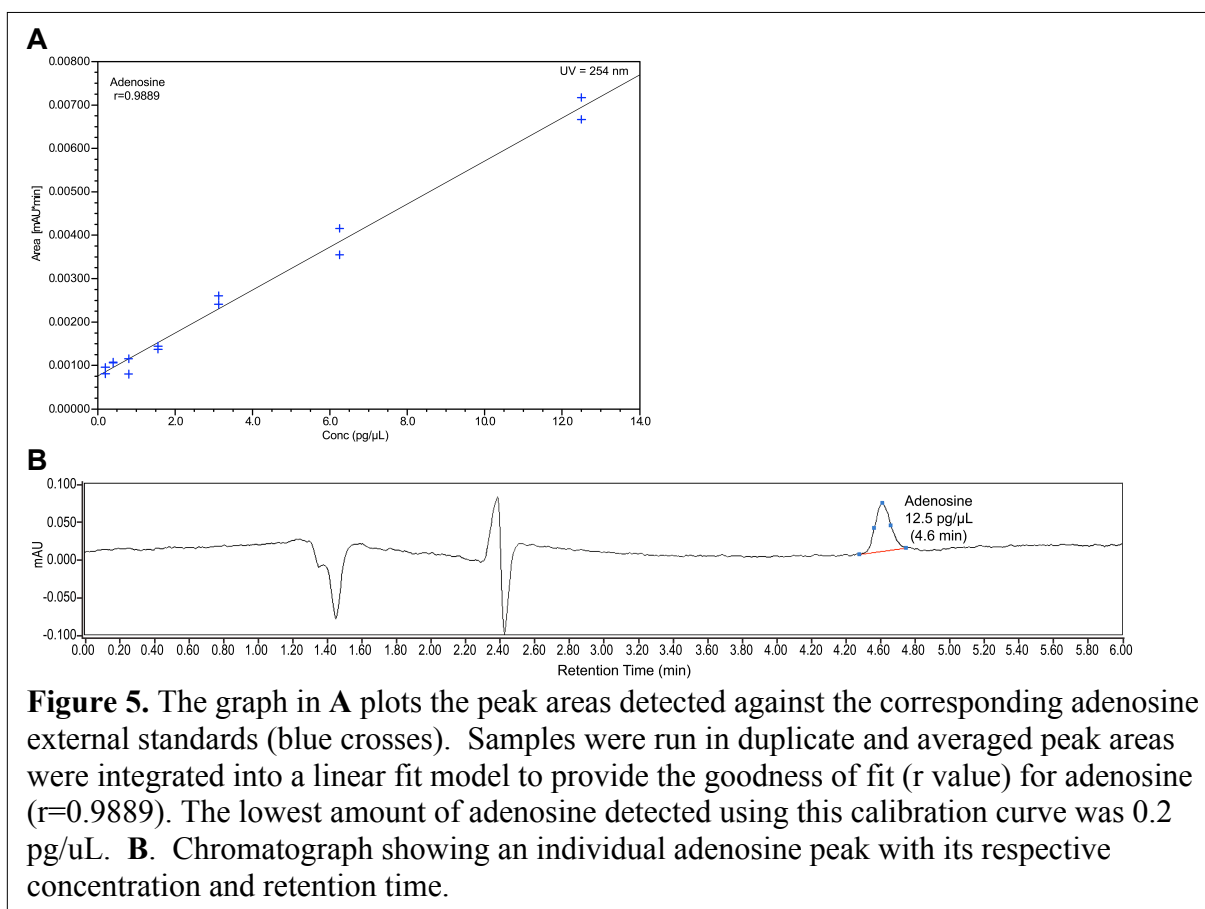
4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis (months 25 to 48).

Progress-Task 4a: Using the procedures described under Tasks 2 and 3 above, 22 mice were administered either ALM (100 mg/kg, i.p.), ZOL (30 mg/kg, i.p.) or vehicle. Half of the rats from each drug treatment condition were then either sleep deprived for 1.5h or left undisturbed, followed by perfusion. Double immunohistochemistry for the marker of functional activity, Fos, and the enzyme neuronal nitric oxide synthase (nNOS; a “sleep-active” cortical neuron population) was performed in coronal sections at the level of the anterior commissure. Counts of double- and single-labeled nNOS-immunoreactive cortical neurons revealed that SD inhibited Fos expression in these sleep-active neurons in all drug conditions (Fig. 4). This finding

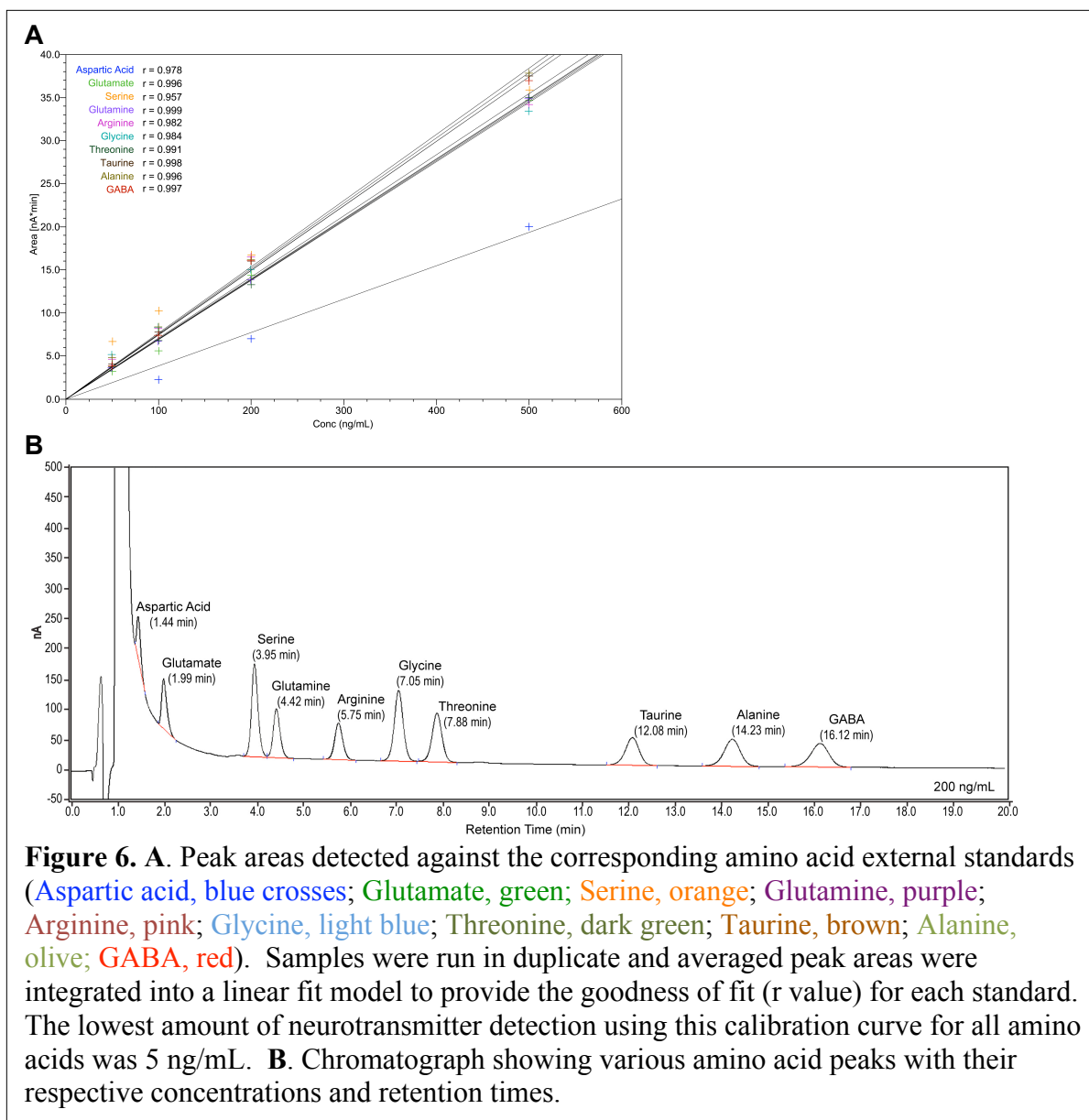
indicates that cortical nNOS neurons are not activated directly by either ALM or ZOL; rather, their activation is coupled to sleep, which is promoted by both compounds.



Progress – Task 4b: As indicated in the last progress report, experiments and infrastructure crucial to Task 4b and 4c were dependent on the completion of a newly-constructed *in vivo* microdialysis facility. We are pleased to report that excellent progress on Task 4b has been made since the facility was completed in November, 2010. Last year, we summarized progress made on validation of 3 HPLC/EC systems, optimized for catecholamine analyses (System 1: norepinephrine, epinephrine, and dopamine; System 2: serotonin; System 3: acetylcholine). We also received approval to purchase an HPLC for determination of GABA, glutamate, glycine and other amino acids, to have capabilities well beyond the scope of the adenosine measurements proposed as Task 4b and 4c in the SOW. This year, we report the functionality of the adenosine and amino acid/GABA HPLC systems. We set up these new two HPLCs -- one to measure adenosine using UV-VIS detection, and the other to measure amino acids and GABA using electrochemical detection -- each optimized for their specific neurotransmitter capabilities. Our initial efforts were directed to validation of internal standards for each system to determine the lower limit of detection *in vivo*.



For the data presented in Figure 5, samples were injected into the HPLC/UV system (Dionex) for the generation of an external standard curve for adenosine (ADO). Adenosine concentrations were dissolved in oxalic acid (1 mM, pH 3.6), and serially diluted to final concentrations in Ringer's solution. The mobile phase consisted of 10 mM Na_2HPO_4 (pH = 4.5) and 7% acetonitrile. Adenosine was carried through with mobile phase, separated through a Kinetex C18 150 x 4.6mm reversed phase column (Phenomenex) at a flow rate of 0.8 mL/min. UV detection was set to 254 nm. The area under the curve of each peak was measured using Chromeleon 6.8.0 software (Dionex, Corp). Figure 4A plots the peak area detected against the corresponding adenosine external standards (blue crosses). Individual samples of known concentrations were run in duplicate and averaged peak areas were integrated into a linear fit model to calculate the goodness of fit for adenosine ($r=0.9889$). The lowest limit of detection for adenosine using this calibration curve was 0.2 pg/μL. Figure 5B presents a chromatograph showing an individual adenosine peak with its respective concentration and retention time.



For the data presented in Figure 6, individual samples were injected into the HPLC/EC system (Dionex) for the generation of an external standard curve for amino acids and GABA (AA/GABA). Amino acid concentrations were made up as a stock solution, dissolved in 50% methanol (MEOH), and serially diluted to final concentrations in 50% MEOH. The mobile phase consisted of 100 mM Na_2HPO_4 , 22% MEOH, and 3.5% acetonitrile, pH 6.75. The amino acid standards were carried through with mobile phase, separated through a Shiseido Capcell Pak C18, 3.0 mm ID x 75 mm, 3 μm reversed phase column from Dionex, and set to a flow rate of 0.4 mL/min. Two electrodes were used, E1; +150 mV, E2; +550 mV, Guard +600 mV. The area under the curve of each peak was measured using Chromeleon 6.8.0 software (Dionex, Corp). Figure 5A plots the peak areas detected against the corresponding amino acid external standards (Aspartic acid, blue crosses; Glutamate, green; Serine, orange; Glutamine, purple; Arginine,

pink; Glycine, light blue; Threonine, dark green; Taurine, brown; Alanine, olive; GABA, red).

Individual samples were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for each standard. The limit of detection using this calibration curve for all amino acids was 5 ng/mL. Figure 6B presents a chromatograph showing various amino acid peaks with their respective concentrations and retention times.

We also report here on the results of the first cohort of animals in Task 4B, to study the effects of oral ALM and ZOL on basal forebrain (BF) adenosine (ADO) release and, in addition, AA/GABA during sleep and wakefulness. We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects GABA_A receptors that are widely distributed in the CNS, we hypothesize that ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used *in vivo* microdialysis and HPLC analyses to examine BF glutamate, GABA, and ADO efflux following oral ZOL (10 mg/kg), ALM (100 mg/kg), or placebo (VEH) combined with behavioral sleep analyses. Male Sprague-Dawley rats (300 ±25 g) used in this study were housed in an ambient-controlled recording room under a 12 h light/12 h dark cycle (lights off at 04:00) with food and water available *ad libitum*. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Experimental design. Male Sprague-Dawley rats (N=9) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as described previously (Morairty et al., 2008). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgical implantation of a unilateral, stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO, glutamate, and GABA. BF coordinates relative to bregma were P -0.3, L +2.0, V -5.0 (Paxinos and Watson, 2009). The electrodes, guide cannula (to permit microdialysis probe placement in the brain of freely-moving rats) and a stainless steel skull screw were fixed to the skull using dental cement. A dummy probe was inserted into the cannula to prevent occlusion prior to the onset of dialysis. For microdialysis, the dummy probe was removed and the microdialysis probe was inserted and locked into position such that the tip of the probe membrane extended 2.0 mm below the edge of the guide cannula.

Rats were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. Animals were also given two separate 1 ml doses of vehicle on two separate days at least one week before the first experimental day. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. At the start of the experiment (4.5 hours into the dark period, ZT16.5), three 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, glutamate, and GABA and baseline EEG, EMG, T_b and LMA were collected to assess behavior. Telemetry data were recorded using DQ ART 3.1 software (Data Sciences Inc., St Paul, MN). Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was subsequently given p.o. to the animals 6h into the dark period (the rats' normal active period) (ZT18), and six additional 30 min samples were collected to assess the effects of the drug on behavior and

neurotransmitter release in the BF. Behavioral measures were simultaneously collected for an additional 1.5 h (total 12 h) post-microdialysis. All samples were collected at 4°C and immediately stored at -80°C until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Behavioral data analyses. Following completion of data collection, sleep-wakefulness was scored in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. EEG and EMG data were scored for waking (W), rapid eye movement sleep (REM), and non-REM (NR). T_{core} and LMA (counts per minute) were analyzed as hourly means. Individual state data were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR and REM onset for each rat was calculated from the time of drug injection. To assess any pharmacological effects on the consolidation of behavioral states, cumulative time spent in W, NR, and REM and the duration and number of bouts of each state was calculated for 3 h following drug administration relative to each 30 min dialysis sample obtained pre- and post-drug administration. Descriptive statistics and analysis of variance (ANOVA) analyses were performed on all behavioral measures. Where ANOVA indicated a probability (*P*) value < 0.05, Dunnett's *post hoc* was used to determine significance between groups.

HPLC analyses. All microdialysis samples were split (10 µL for ADO, 20 µL for AA/GABA) into two vials for HPLC analyses. ADO samples were separated by reverse-phase HPLC with a Kinetic column (Phenomenex C18 150 x 4.6mm) and monitored at 254 nm by UV. The mobile phase consisted of 10 mM Na₂HPO₄ (pH = 4.5), and 7% acetonitrile and was set to a flow rate of 0.8 mL/min. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex, Corp). Amino acids, glutamate and GABA were assayed using HPLC-EC. The mobile phase consisted of 100 mM Na₂HPO₄, 22% ME OH, and 3.5% acetonitrile, pH 6.75 and set to a flow rate of 0.4 mL/min. The amino acids were detected by precolumn derivitization using O-phthalaldehyde (OPA) and 2-mercaptoethanol (βME) with automation at 4°C, 2 min prior to injection into the HPLC. Separation was achieved with a reversed-phase column by Shiseido (Capcell Pak C18, 3.0 mm ID x 75 mm, 3 µm) and electrically detected at the following potentials; E1; +150 mV, E2; +550 mV, Guard +600 mV. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex Corp). Descriptive statistics and a two-way ANOVA were used to determine the effect of sleep-wake states on ADO, glutamate, and GABA release. Post hoc comparisons were performed using Tukey's multiple pairwise comparison tests. A probability (*P*) value < 0.05 was used to evaluate the significance of all statistical tests.

Behavioral State Results. To date, a total of 9 rats contributed to the current set of results. As illustrated in Figure 7, representative hypnograms show the effects of VEH, ZOL (10 mg/kg, p.o.), and ALM (100 mg/kg, p.o.) administration on individual animals' sleep-wake architecture for each treatment condition.

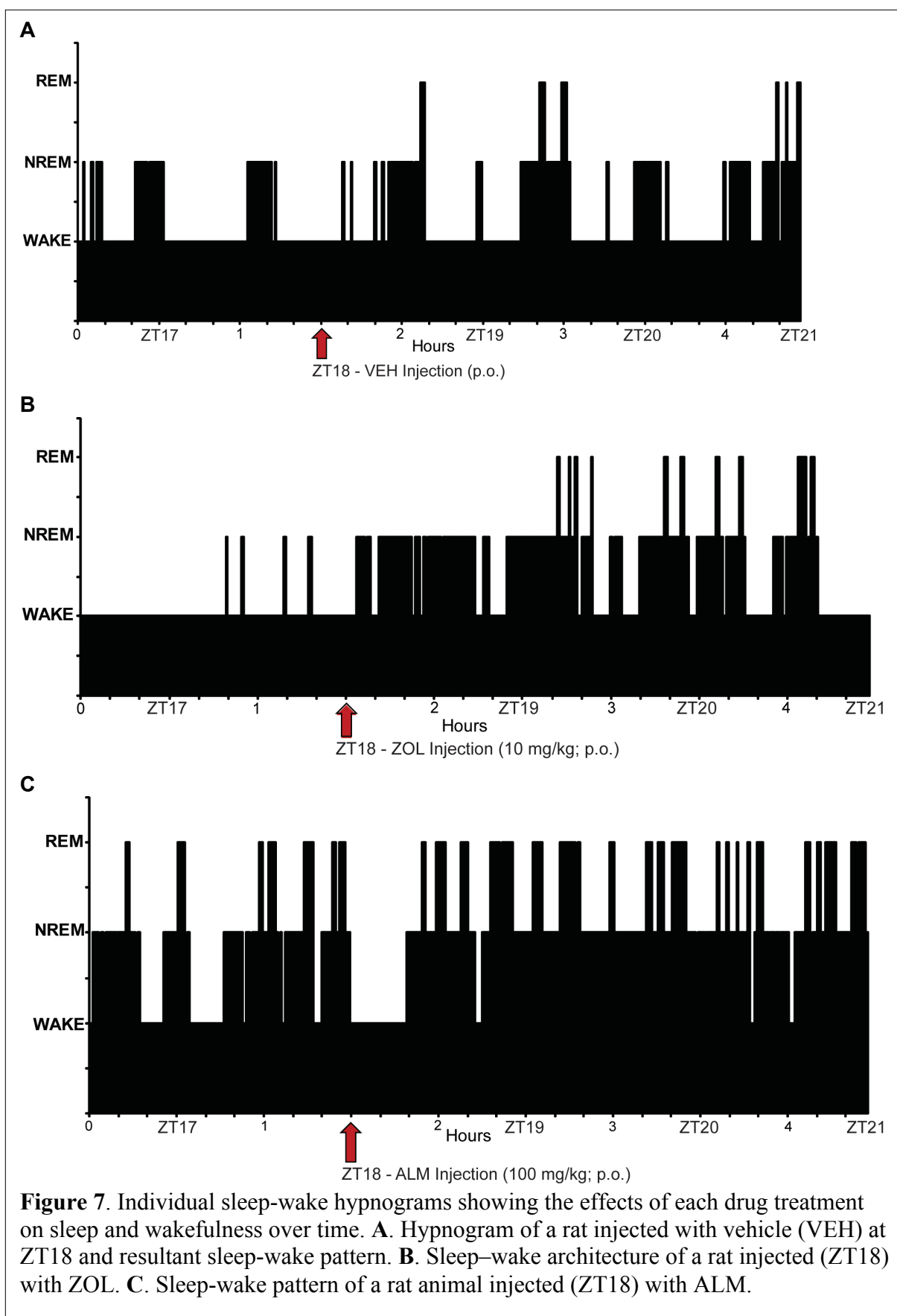


Table 1 summarizes the effects of VEH, ZOL, and ALM administration (p.o.) on sleep-wake parameters for each treatment condition following drug delivery. ANOVA revealed a significant drug effect on wake, NR, and R states (* $p < 0.05$). Dunnett's *post-hoc* analyses showed that ZOL and ALM had significant effects on the total amount of time spent in Wake and NR compared to VEH. In addition, ALM significantly increased (* $p < 0.05$) mean Wake duration, and the number of NR and R bouts compared to VEH control animals.

Table 1. Sleep-wake parameters in VEH, ZOL-, and ALM- treated rats post-drug administration. Values (means \pm SEM) are calculated for a 3 h period during the dark phase.

Sleep/Wake Parameter	VEH	ZOL (10 mg/kg)	ALM (100 mg/kg)
Cumulative Wake (min)	144.2 (11.6)	96.2 (9.3)*	61.7 (5.2)*
No. of Wake Bouts	20.0 (4.2)	22.2 (2.5)	25.4 (1.5)
Mean Duration of Wake Bouts (min)	8.3 (2.8)	4.6 (0.8)	2.4 (0.1)*
Cumulative NREM (min)	33.8 (9.3)	75.4 (7.6)*	87.7 (2.6)*
No. of NREM Bouts	20.3 (4.4)	25.6 (2.7)	38.0 (2.3)*
Mean Duration of NREM Bouts (min)	1.6 (0.3)	2.9 (0.2)*	2.3 (0.2)
Latency to NREM (min)	9.6 (5.8)	9.6 (3.8)	17.0 (2.0)
Cumulative REM (min)	4.94 (2.5)	11.2 (2.1)	33.5 (3.5)*
No. of REM Bouts	5.0 (2.6)	10.0 (2.3)	24.2 (3.2)*
Mean Duration of REM Bouts (min)	0.7 (0.3)	1.3 (0.3)	1.4 (0.2)
Latency to REM (min)	22.3 (11.5)	44.6 (13.4)	26.2 (1.2)

* $p < 0.05$ determined by 1-way ANOVA with Dunnett's multiple comparison *post hoc* analyses.

Neurotransmitter Release Results. The results to date are based on a total of 3,390 min of microdialysis sampling across the sleep-wake cycle from 9 rats. Dialysis samples were split into two and processed for both ADO and glutamate/GABA content. Two-way ANOVA revealed a significant drug \times state interaction for all neurotransmitters. Tukey's *post hoc* comparisons showed that oral ZOL (* $p < 0.05$) caused a significant increase in BF glutamate release (Figure 8A) when sampling during 30 min timeframes comprised of wakefulness compared to ALM or VEH. Oral ALM (* $p < 0.05$) significantly increased BF glutamate (Figure 8B) under sampling conditions that showed a mixture of Wake, NR, and R. On the other hand, ALM decreased BF glutamate (Figure 8C) during sampling when only NREM/REM sleep occurred during the compared to ZOL. Since no 30 min consolidated NREM/REM period occurred following VEH, the comparison between VEH and drug treatments could not be made.

Oral ALM concurrently decreased BF GABA release as demonstrated by Tukey's *post-hoc* (* $p < 0.05$) during dialysis sampling periods when the animals cycled between Wake, NR, and R (Figure 9B). On the other hand, GABA release was significantly higher in the ALM (* $p < 0.05$) condition compared to ZOL during NREM/REM cycling and the corresponding collection timeframes (Figure 9C).

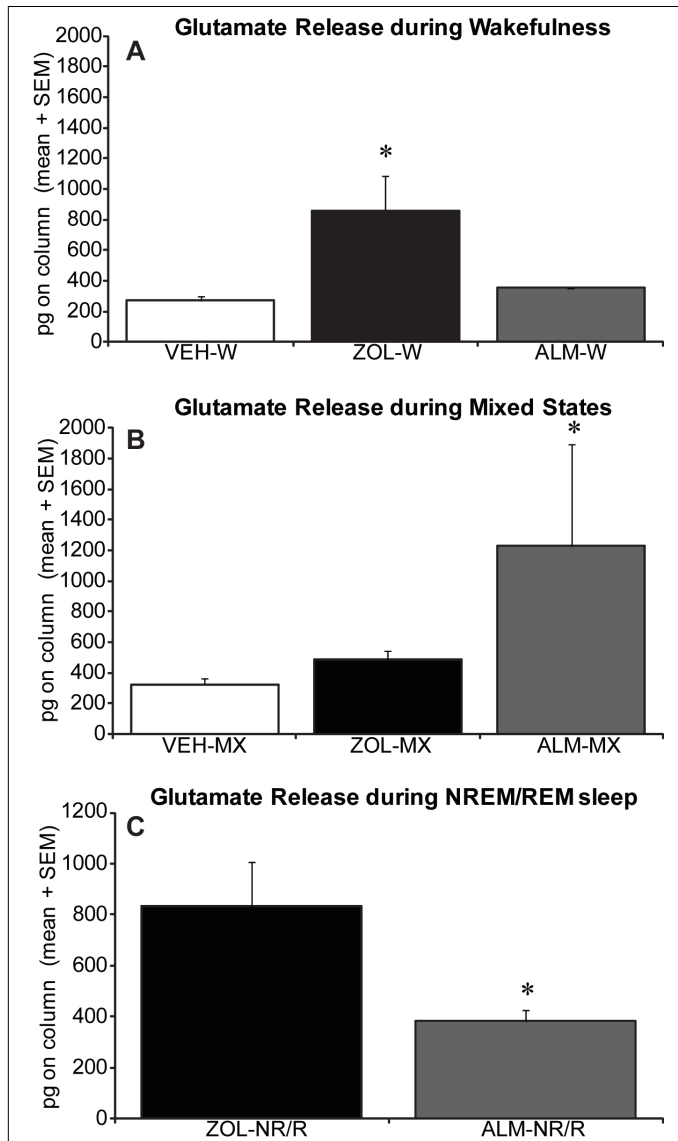


Figure 8. Glutamate release changes during sleep-wakefulness as a function of drug treatment. **A.** Tukey's *post-hoc* comparisons showed that ZOL increased glutamate release when sampling during states of wakefulness ($*p<0.05$) compared to ALM. **B.** Glutamate release under sampling conditions that showed a mixture of Wake, NREM (NR), and REM (R), was significantly higher in the ALM ($*p<0.05$) condition compared to ZOL. **C.** ALM caused a significant decrease in BF glutamate during NR/R and the corresponding collection timeframes compared to ZOL or VEH ($*p<0.05$).

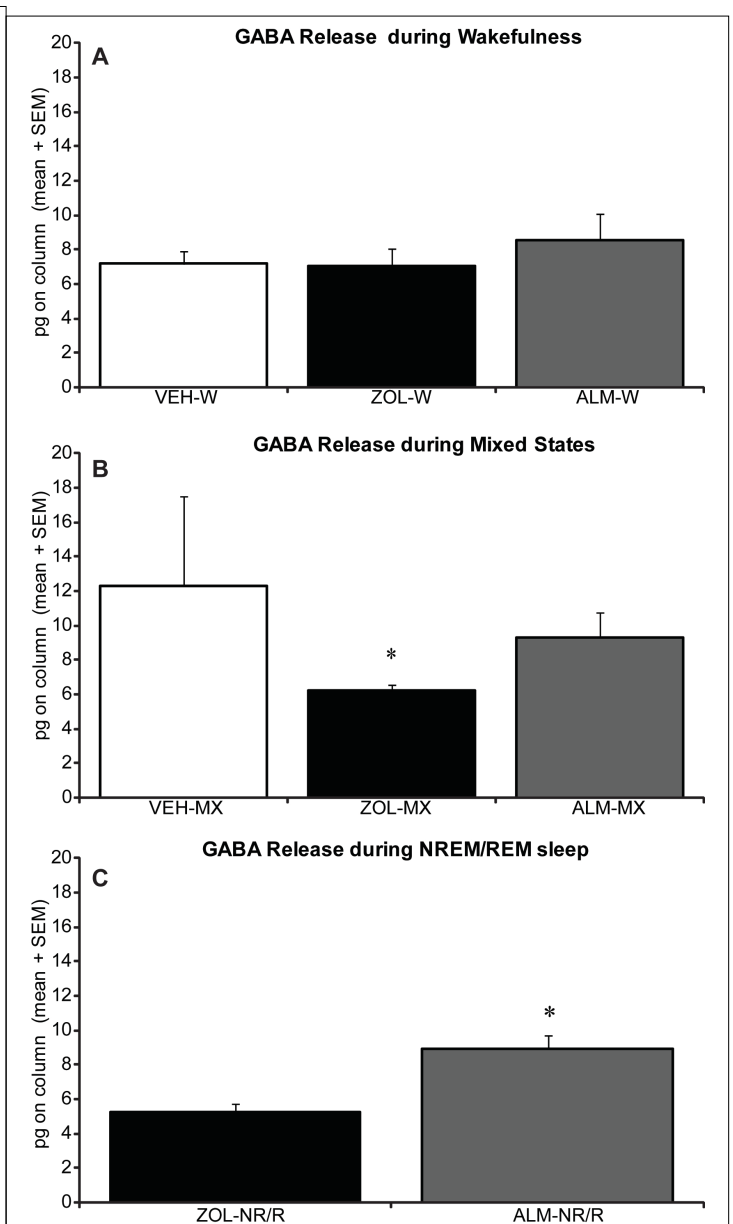
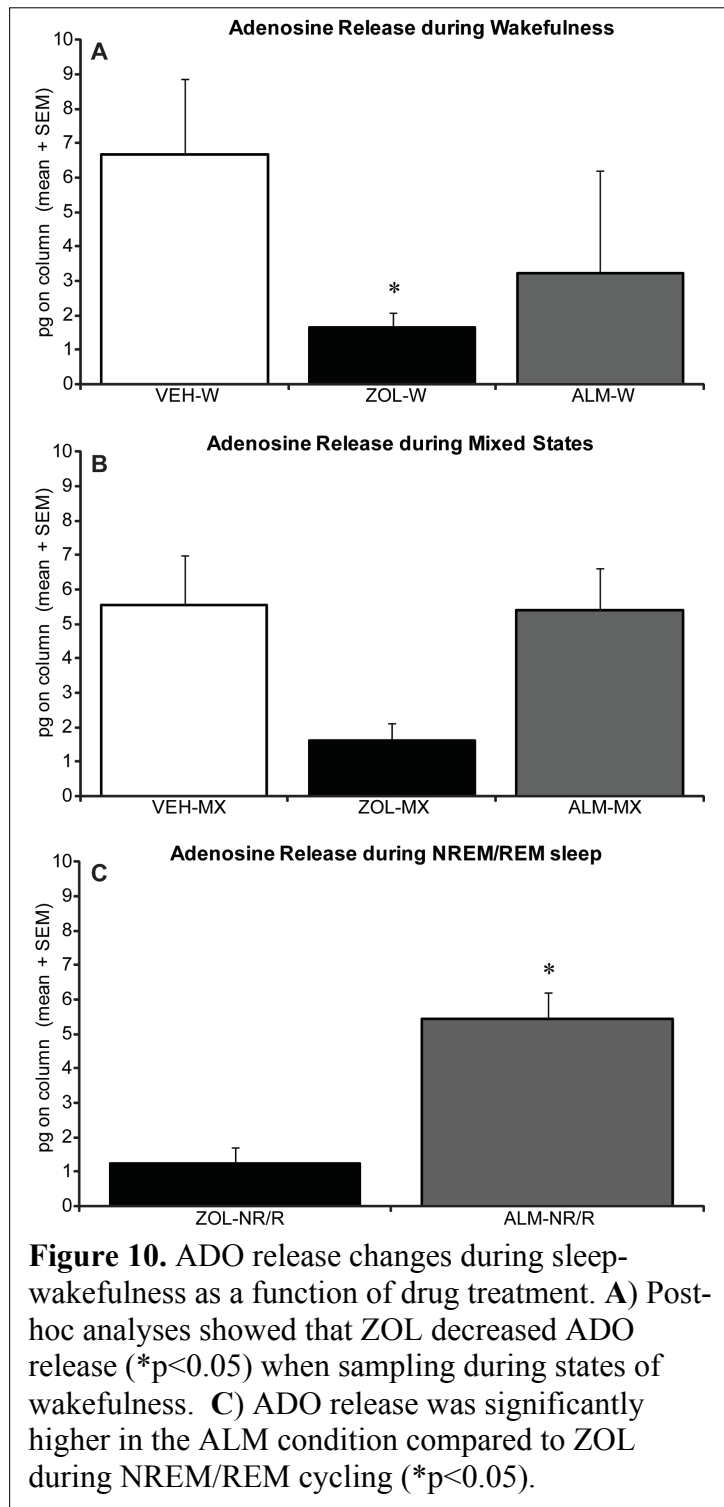


Figure 9. GABA release changes during sleep-wakefulness as a function of drug treatment. In **B**, *post hoc* analyses showed that ZOL decreased GABA release during sampling while the animals presented mixed states of Wake, NR, and R. **C.** GABA release was significantly higher in the ALM condition compared to ZOL during NREM/REM cycling and the corresponding collection timeframes ($*p<0.05$).



Analyses of ADO levels revealed that oral ZOL caused a significant decrease in BF ADO release (Figure 10A) when sampling during 30 min timeframes comprised of wakefulness compared to ALM or VEH (* $p < 0.05$; Tukey's *post hoc* test). However, oral ALM significantly increased BF ADO (Figure 10C; * $p < 0.05$) under sampling conditions where only NREM/REM cycling occurred during the corresponding collection timeframes compared to ZOL or VEH. These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

Task 5: *Test the hypothesis that neural gene expression that occurs ALM-induced sleep more closely resembles that of spontaneous sleep than does ZOL-induced sleep.*

5a. Comparison of ALM and ZOL effects on expression of plasticity-related genes (months 37 to 48).

5b. Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep (months 37 to 48).

Progress: None anticipated prior to Year 3.

Plans for Year 3:

Task 2: Tasks 2a and 2b will be completed. For Task 2c, the assay will be established and data collection will begin. The order for the testing chamber will be placed in August, 2011.

Task 3: Immunostaining of brains for other wake-active neuronal populations (cholinergic, serotonergic and noradrenergic neurons) is ongoing. Michael Schwartz, Ph.D. will join our staff as a Research Scientist on August 1, 2011 to lead the efforts on Task 3b in Year 3. He will also initiate acquisition and breeding of mice as necessary to execute Task 3c, which will likely occur primarily in Year 4.

Task 4: We will continue collecting data from another cohort of animals in order to reach our proposed statistical power of 8 rats per treatment group in Task 4b. The behavioral and neurotransmitter analyses will then be submitted for publication. The study design for Task 4b, BF ADO release in response ALM and ZOL by local dialysis will require some experimental modifications as we have determined that neither ALM nor ZOL readily pass across the dialysis membrane. The drug characteristics and permeation rate of ALM and ZOL depend upon the concentration of drug, the oil/water partition coefficient of drug, and the surface area of the dialysis membrane. To permit sufficient compound of either ALM or ZOL to flow across the membrane, we've surmised that (1) neither ALM nor ZOL would have relative efficacy on release or behavior at the minimum concentration, (2) these compounds are not readily dissolved in water, and (3) the surface area of the dialysis membrane is limited by the area of the brain structure (small region within the BF) in which we want the drugs to freely diffuse across without affecting other major brain regions. Thus, we propose to microinject ALM and ZOL (using several different concentrations) into the BF and collect microdialysis samples from the sleep-active cortical regions (Gerashchenko et al, 2008) of freely-moving animals instead and assess ADO, glutamate and GABA release along with simultaneous behavioral measures.

Task 5: As follow up to Dr. Kilduff's July 22, 2011 email and July 25, 2011 letter to LCDR Mark D. Clayton, Ph.D., CDMRP Science Officer, we request permission to modify the focus of Task 5 from gene expression studies to optogenetics and *in vivo* cellular neurophysiology, which we believe would be more informative technical approaches with regard to the overall theme of "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance." As is evident in this report, our research efforts involve systems physiology/pharmacology approaches to understand how the Hcrtr receptor antagonist ALM is producing sleep without (apparently) impairing performance. To advance understanding of this question, we believe that optogenetic and *in vivo* cellular neurophysiology approaches would complement our ongoing behavioral, *in vivo* physiology, functional neuroanatomical and neurochemical approaches. In particular, the recent paper published in *Journal of Neuroscience* by Tsunematsu et al. (*J. Neurosci.* 2011; 31:10529-10539) presents a paradox with respect to

understanding how ALM induces sleep since, in that study, optogenetic silencing of the Hcrt cells induces sleep only during the day but not at night. Conversely, previous studies have shown that optogenetic activation of the Hcrt cells through the blue light-sensitive channelrhodopsin (ChR2) protein during sleep reduces the latency to awakening (Adamantidis et al., 2007; Carter et al., 2009).

Using a combination of optogenetics and *in vivo* cellular neurophysiology, we propose to address the following questions related to the overall theme of “Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance”:

1) **Is activation of the Hcrt system sufficient to induce arousal in the presence of ALM?** As is evident in Figure 2, ALM-treated rats are able to arouse from ALM-induced sleep and perform at a high level, at least in this spatial reference memory task. The mechanism underlying the arousal from sleep is unlikely to be mediated through the Hcrt peptides because Hcrt receptors should be blocked by the presence of ALM. However, Hcrt neurons also release glutamate and perhaps other unknown neurotransmitters. To address this question, we will induce arousal from spontaneous vs. ALM-induced sleep by optogenetic stimulation of the Hcrt neurons in a transgenic mouse that specifically expresses the blue light-sensitive ChR2 in Hcrt neurons (*orexin/ChR2* mice). If the Hcrt system is truly involved in arousal from sleep, ALM should block optogenetic-induced awakenings. If optogenetic-induced awakenings persist in the presence of ALM, we can conclude that other neurotransmitters released by the Hcrt neurons are the likely cause of the arousal. These data will complement the behavioral studies being conducted in Task 2.

2) **How does ALM affect the activity of subcortical sites downstream from the Hcrt neurons?** Although the optogenetic papers cited above as well as others in the literature review in our original proposal focus on the importance of the Hcrt neurons for maintenance of wakefulness and arousal from sleep, Hcrt neurons are part of a larger network that underlie sleep/wake control. To this point, there is no information of the effects of ALM on these “downstream” Hcrt efferent projection sites. We hypothesize that ALM will blunt optogenetically-induced neural activation in subcortical Hcrt projection sites such as the serotonergic dorsal raphe nucleus (DR) and noradrenergic locus coeruleus (LC) whereas ZOL will not. To address this hypothesis, we will conduct optogenetic stimulation of Hcrt neurons in *orexin/ChR2* mice while simultaneously conducting multiunit recordings from efferent projection sites such as the DR and LC. These data will complement the functional neuroanatomical studies being conducted in Task 3.

3) **How does Hcrt neural activity affect cortical function?** In order to appropriately perform cognitive tasks, the cerebral cortex must be engaged and unimpaired. The data in Figure 2 indicate that performance on a hippocampal-mediated spatial reference memory task is unimpaired in the presence of ALM. Task 2c is intended to compare cortical function in the presence of ALM vs. ZOL. Although both Hcrt receptors 1 and 2 are differentially expressed at low levels within the cortex (Marcus et al., 2001), to this point, it is unknown whether ALM affects cortical neuron activity. We hypothesize that ALM will directly affect the activity of a subset of cortical interneurons present in cortical layers 5 and 6. To address this hypothesis, we will compare the effects of ALM and ZOL on cortical firing and local field potentials using a 64 channel cortical array and a 16 channel vertical probe during the sleep/wake cycle and during optogenetic manipulation of Hcrt neurons. We expect that ZOL will have a generalized inhibitory effect on cortical neuron activity whereas ALM will only affect a subset of cortical

neurons. The results of these studies will assist in the interpretation of the behavioral studies being conducted in Task 2c, the functional neuroanatomical studies in Task 4a and the revised microdialysis studies in Task 4c.

Budgetary implications: Implementation of the optogenetic and *in vivo* cellular neurophysiology approaches proposed here in lieu of the microarray approaches previously proposed will have budgetary implications. Task 5 as originally proposed was scheduled to start in Year 4 whereas we would like to initiate the revised Task 5 in Year 3. In a 24 June 2011 email, Ms. Jennifer Shankle, USAMRAA Grant Specialist, pointed out that we have been underexpending funds on USAMRAA Grant W81XWH-09-2-0081 to date. As explained in Dr. Kilduff's 27 June 2011 response, this underexpenditure was a result of the delay in initiating Tasks during Year 1 while laboratory construction was ongoing. SRI requests approval to spend our current funding to initiate work on the revised Task 5 during Year 3. The funding we currently have available is adequate to complete the currently approved Tasks and to initiate Task 5 in Year 3 and we currently have trained staff on hand who can conduct the proposed studies. We will need to reallocate funds within our current budget as these new approaches will necessitate the purchase of some equipment. Because of the synergism that we expect to result from implementation of these approaches with the approaches used in execution of Tasks 2-4, we believe that this budget reallocation is not only highly justified in terms of achieving the overall goals of this proposal, but that integration of these technical approaches will enable us to conduct higher quality science.

KEY RESEARCH ACCOMPLISHMENTS

- Occupation of a new laboratory for behavioral performance assessment and microdialysis sampling in November, 2010.
- Full system installation of all hardware equipment and software, and validated communication and automation capabilities for two HPLCs: one to measure adenosine and the other to measure amino acids.
- Establishment of a spatial reference memory test and demonstration that ZOL impairs performance on this test whereas ALM does not (Figure 2).
- Preliminary results obtained indicating that the wake-active Hcrt neurons could be activated in the presence of ALM but not in the presence of ZOL (Figure 3).
- Determination that both ALM and ZOL activated a sleep-active cortical neuron population (Figure 4).
- Establishment of limits of detection for the two new HPLCs (Figures 5-6).
- Determination of the effect of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figures 8-10).
- Submission of two abstracts to be presented at the annual Society for Neuroscience meeting to be held in Washington, D.C. in Nov 2011.

REPORTABLE OUTCOMES

Abstracts submitted for Society for Neuroscience meeting (Washington, DC, Nov 12-16, 2011)

- L Dittrich, S Morairty, D Warrier, A Wilk, K Silveira, TS Kilduff, “The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake”.

- J. Vazquez-DeRose, A. Nguyen, and T. S. Kilduff. “Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat”.

CONCLUSION

Preclinical data indicate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. In Year 2, we have found that both the benzodiazepine receptor agonist ZOL and the Hcrtr receptor antagonist almorexant (ALM) induced sleep in rodents but ALM did not impair performance in a spatial reference memory test whereas ZOL did. The lack of impairment may, in part, be due to the fact that the wake-active Hcrtr neurons could be activated in the presence of ALM but not in the presence of ZOL. In contrast, a sleep-active cortical neuron population was equally activated by ALM and ZOL. During NREM/REM sleep, ALM caused a significant decrease in basal forebrain (BF) glutamate and concurrently increased BF GABA and adenosine compared to ZOL or VEH. These results are consistent with the hypothesis that disfacilitation of wake-promoting systems by ALM results in less functional impairment than the general inhibition of neural activity produced by ZOL.

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APPENDICES

None.

Appendix 3: Revised Statement of Work

STATEMENT OF WORK

Revised September, 2011

Initiating PI: Thomas C. Neylan, M.D. **Collaborators:** Steven Batki, M.D., Charles R. Marmar, M.D., Shannon McCaslin, Ph.D., Angela Waldrop, Ph.D., *Nancy J. Wessenden, Ph.D., Thomas Balkin, Ph.D.*

Institution: Northern California Institute for Research and Education

Address: 4150 Clement Street, San Francisco, CA 94121

Human Use: Yes, **Anatomical Samples:** No, **Animal Use:** No

Partnering PI: Thomas S. Kilduff, Ph.D. **Collaborators:** Stephen Morairty, Ph.D., Jacqueline Vazquez-DeRose, Ph.D.

Institution: SRI International

Address: 333 Ravenswood Avenue, Menlo Park, CA 94025

Human Use: No, **Anatomical Samples:** Yes, **Animal Use:** Yes

The Initiating PI would like to request the following revisions:

- 1.) Some of the neuropsychological assessments listed in the original SOW have been replaced with assessments which tap into the same militarily relevant neuropsychological processes. Please refer to Page 2 for details.
- 2.) The neuropsychological testing schedule has been finalized and is included in a table on Page 3.

The Partnering PI would like to request the following revisions:

- 1.) Omission of Tasks 5a (Comparison of ALM and ZOL effects on expression of plasticity-related genes) and 5b (Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep). Please refer to Pages 4-6.
- 2.) Addition of tasks 6a (Determine whether activation of the Hcrt system induces arousal in the presence of ALM vs. ZOL), 6b (Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons), and 6c (Determine how ALM and ZOL affect the activity of cortical neurons). Please refer to Page 6.
- 3.) Addition of the following detail in the “General Methods” section (refer to Page 8):
 - a. *In vitro* electrophysiological recordings
 - b. *In vivo* extracellular recordings
 - c. Optogenetic illumination *in vivo*.

Description of Work to be done

We will conduct a multi-center integrated translational study of the effect of a novel hypocretin/orexin antagonist (ALM) to a standard hypnotic (zolpidem-ZOL) and placebo (PBO) on neurocognitive performance at peak concentration post dosing. The human study component (Task 1) will establish if ALM is superior to ZOL regarding neurocognitive side effects. The animal studies (Tasks 2-5) will be conducted to define the neural circuitry that underlies the activity of these compounds, and the effects of these compounds on biomarkers associated with normal sleep. The overall hypothesis is that hypocretin (Hcrt) antagonists produce fewer functional impairments than benzodiazepine receptor agonists (BzRA) because BzRAs causes a general inhibition of neural activity whereas Hcrt specifically disfacilitates wake-promoting systems.

Task 1. *Test the hypothesis that healthy human subjects receiving ZOL 10mg will show greater impairment in neurocognitive performance compared to subjects receiving PBO or the 2 doses (100mg, 200mg) of ALM.*

Design: The human study will entail in-laboratory assessment using sensitive, widely-used metrics of militarily relevant aspects of neurocognitive performance following administration of 2 different dosages of almorexant versus the currently recommended dose of zolpidem or placebo.

Human subjects: Study subjects (n = 200) will be evaluated at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and housed in the Clinical Research Center (CRC) of the Clinical and Translational Science Institute (CTSI) at the University of California, San Francisco (UCSF). The project will be administered by the Northern California Institute for Research and Education (NCIRE) which is affiliated with both UCSF and the SFDVAMC.

Methods: Neurocognitive testing will follow 1 week of sleep-wake monitoring with actigraphy, 2 adaptation nights, blinded administration of a sleep-inducing agent, neurocognitive performance testing, and 1 night of recovery sleep in the laboratories at UCSF.

Outcomes, products and deliverables: It is predicted that both doses of almorexant will have less negative impact on neurocognitive performance than zolpidem. If so, these results will show that sleep-inducing agents targeting hypocretin/orexin receptors have a superior safety profile relative to those agents that act as BZ receptor agonists – information that will ultimately allow these agents to be used under operational conditions.

Timeline: 9-12 months: production and all approvals of human use protocols, purchase of equipment; 12-36 months: recruitment and completion of data collection on 200 volunteers; 36-48 months: complete neurocognitive task scoring, sleep scoring and data analyses, final report/manuscripts written and submitted.

Responsible Individual: Thomas C. Neylan, M.D.

Timelines/Milestones Summary Table

TIMETABLE FOR THE RESEARCH PROGRAM		YR1	YR2	YR3	YR4
Task #1:	Determine neurocognitive effects of Almorexant versus Zolpidem				
Subtask #1.	Write protocol (4 months)				
Subtask #2.	Obtain scientific and human use approvals (6 months)				
Subtask #3.	Purchase study related equipment/supplies (4 months)				
Subtask #4.	Train laboratory personnel				
Subtask #5.	Collect data on 200 volunteers (approximately 22 participants will be enrolled each quarter)				
Subtask #6.	Score and analyze data				
Subtask #7.	Write/publish final report				

Tests to be administered relative to drug administration are listed in the Table below. Tasks were selected based on their demonstrated sensitivity to sleep-inducing agents (Stanford Sleepiness Scale, Maintenance of Wakefulness Test - both of which are widely accepted in the sleep research community as “gold standard” metrics of subjective and objective sleepiness, respectively) or because they index militarily relevant aspects of neurocognitive function. The following are widely accepted in the neuropsychology community as tapping listed neurocognitive processes: Rey Auditory Verbal Learning Test = short term and delayed auditory memory and learning; Paired Associates Task= learning and cued recall, Psychomotor Vigilance Task= sustained

attention and reaction time; Tower Test from Delis-Kaplan Executive Function System = executive function; Continuous Performance Test = attention, working memory, executive function; Stroop Color-Word Test = ability to inhibit pre-potent responses; Digit Span Subtest of the WAIS-IV = attention and working memory; Grooved Pegboard = manipulative dexterity. These tests will be administered according to the testing schedule below:

Schedule of Neuropsychological Testing

Testing Day Time	Test	Avg. Admin Time
1000 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
1030hrs	Psychomotor Vigilance Test	11 min
1040 hrs - 1200hrs	Break	1 hr 20 min
1200 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
1230 - 1330hrs	Lunch	1 hr
1330 hrs	Rey Auditory Verbal Learning Test (List 1)	10 min
	Continuous Performance Task	16 min
	Symptom Checklist	5 min
1400-1500	Break	1 hour
1500 - 1510hrs	Dosing	10 min
1510 hrs	Psychomotor Vigilance Test	10 min
1520 - 1530hrs	Break	10 min
1530 hrs	Stanford Sleepiness and Maintenance of Wake	25 min
1555 hrs	Rey Auditory Verbal Learning Test (Delayed)	4 min
	Continuous Performance Task	16 min
	Paired-Associates	7 minutes
	Rey Auditory Verbal Learning Test (List 2)	10 min
	Symptom Checklist	1 min
	Stroop Color-Word Test	5 min
	Grooved Pegboard	8 min
1645hrs	Psychomotor Vigilance Test	15min
1700hrs	Towers	20 min
	Digit Span	6 min
1730hrs	Stanford Sleepiness and Maintenance of Wake	30 min
	Symptom Checklist	1 min
1805 - 1910 hrs	Dinner	65 min
1910 hrs	Psychomotor Vigilance Test	10 min
1920 hrs - 1930 hrs	Break	10 min
1930 hrs	Stanford Sleepiness and Maintenance of Wake	30 min
2000 hrs	Paired-Associates (Delayed)	5 min
2005 hrs	Symptom Checklist	5 min
2010 hrs	Rey Auditory Verbal Learning Test (List 3)	10 min
2020hrs - 2105hrs	Break	45 min
2105 hrs	Psychomotor Vigilance Test	10 min
2115 - 2130 hrs	Break	15 min
2130 hrs	Stanford Sleepiness and Maintenance of Wake	30 min

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

2a. Assessment of Almorexant effects on spatial reference memory in rats (months 1 to 12).

2b. Assessment of Almorexant effects on spatial working memory in rats (months 1 to 12).

2c. Assessment of Almorexant effects on psychomotor vigilance in rats (months 13 to 24).

Responsible Individual: Stephen Morairty, Ph.D.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*

3a. Double-label immunohistochemistry with Fos and phenotypic markers (months 1 to 12).

3b. Assessment of hypnotic efficacy in saporin-lesioned rats (months 13 to 24).

3c. Assessment of hypnotic efficacy in transgenic mice (months 25 to 36).

Responsible Individual: Thomas S. Kilduff, Ph.D.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*

4a. Effects of ALM and ZOL on sleep-active brain areas (months 1 to 12).

Responsible Individual: Thomas S. Kilduff, Ph.D.

4b. BF adenosine (ADO) release in response to oral ALM and ZOL (months 1 to 24).

4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis (months 25 to 48).

Responsible Individual: Jacqueline Vazquez-DeRose, Ph.D.

~~**Task 5:** *Test the hypothesis that neural gene expression that occurs ALM-induced sleep more closely resembles that of spontaneous sleep than does ZOL-induced sleep.*~~

~~5a. Comparison of ALM and ZOL effects on expression of plasticity related genes (months 37 to 48).~~

~~5b. Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep (months 37 to 48).~~

~~Responsible Individual: Thomas S. Kilduff, Ph.D.~~

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL (months 25 to 36).

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons (months 37 to 48).

6c. Determine how ALM and ZOL affect the activity of cortical neurons (months 37 to 48).

Responsible Individual: Thomas S. Kilduff, Ph.D.

Task#2	TASK/Subtask	Yr 1	Yr 2	Yr 3	Yr 4
Subtask #2a	Assessment of ALM Effects on Spatial Reference Memory Animal use approval, experiment, analysis 80 SD rats Milestone: Submission of report	X XXX			
Subtask #2b	Assessment of ALM Effects on Spatial Working Memory Milestone: Submission of report	XXX			
Subtask #2c	Assessment of ALM Effects on Psychomotor Vigilance Animal use approval, experiment, analysis 15 SD rats Milestone: Submission of report		X XXX		
Task#3	TASK/Subtask	Yr 1	Yr 2	Yr 3	Yr 4
Subtask #3a	Double-label immunohistochemistry with Fos and phenotypic markers Experiment and analysis 24 SD rats Milestone: Submission of report	X XXX			
Subtask #3b1	Assessment of hypnotic efficacy in TMN-lesioned rats Experiment and analysis 24 SD rats Milestone: Submission of report		X XXX		
Subtask #3b2	Assessment of hypnotic efficacy in LC-lesioned rats Experiment and analysis 24 SD rats Milestone: Submission of report		X XXX		
Subtask #3b3	Assessment of hypnotic efficacy in BF-lesioned rats Experiment and analysis 26 SD rats Milestone: Submission of report		X XXX		
Subtask #3c	Assessment of hypnotic efficacy in transgenic mice Experiment and analysis 48 mice (3 strains + 3 controls; 8/group) Milestone: Submission of report			X XXX	
Task#4	TASK/Subtask	Yr 1	Yr 2	Yr 3	Yr 4
Subtask #4a	Effects of ALM and ZOL on sleep-active brain areas Experiment and analysis 24 SD rats Milestone: Submission of report	X XXX			
Subtask #4b	BF adenosine (ADO) release in response to oral ALM and ZOL Experiment and analysis 16 rats/microdialysis study Milestone: Submission of report	X	X XXX		
Subtask #4c	BF adenosine (ADO) release in response to ALM and ZOL by dialysis Experiment and analysis Milestone: Submission of report			X	X XXX
Task#5	TASK/Subtask	Yr 1	Yr 2	Yr 3	Yr 4
Subtask #5a	Comparison of ALM and ZOL effects on expression of plasticity-related genes				

	Experiment and analysis 27 SD rats	-	-	-	X
	Milestone: Submission of report	-	-	-	XXX
Subtask #5b	Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep				
	Experiment and analysis 27 SD rats	-	-	-	X
	Milestone: Submission of report	-	-	-	XXX
Task#6	TASK/Subtask	Yr 1	Yr 2	Yr 3	Yr 4
Subtask #6a	Determine whether activation of the Hcrt system induces arousal in the presence of ALM vs. ZOL				
	Optogenetic activation of Hcrt neurons in ALM vs. ZOL-treated rodents			X	
	Milestone: Submission of report			XXX	
Subtask #6b	Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons				
	Optogenetic activation of Hcrt neurons and recording in the raphe nuclei or locus coeruleus of ALM vs. ZOL-treated rodents				X
	Milestone: Submission of report				XXX
Subtask #6c	Determine how ALM and ZOL affect the activity of cortical neurons				
	Optogenetic activation of Hcrt neurons and recording in cerebral cortex of ALM vs. ZOL-treated rodents				X
	Milestone: Submission of report				XXX

General Methods

Subjects. The proposed experiments will utilize Sprague-Dawley rats and mice of three strains: DBH knockout (KO) mice (Hunsley and Palmiter, 2003, 2004), histidine decarboxylase (HDC) KO mice (Parmentier et al., 2002), and *Lmx1b* knockout (central serotonin-deficient mice (Hodges et al., 2008). Animals will be housed in a temperature-controlled recording room under a 12/12 light/dark cycle and have food and water available ad libitum. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions will be monitored continuously via computer. Animals will be inspected daily in accordance with AAALAC and SRI guidelines. All experimental procedures involving animals are approved by SRI International's Institutional Animal Care and Use Committee (IACUC) and will be in accordance with National Institute of Health (NIH) guidelines.

Morris Water Maze. All water maze tasks will occur in a pool 72" in diameter and 25" in depth, containing opaque water at 26±2°C. Distinctive spatial cues (e.g. signs, laboratory furniture) will be clearly visible from within the pool. A 10 cm diameter platform will be submerged approximately 1 cm below the surface of the water. To cue the platform location during the working memory trials, a flag will be attached to the platform to extend 10 cm above the water surface. Performance will be monitored with a video tracking system (MED Associates).

Rat Psychomotor Vigilance Task (rPVT). The rPVT tests will occur in operant chambers (MED Associates) with infrared detection beams in front of the water dispenser. Rats will be motivated to perform the operant rPVT task for water reinforcements by having water unavailable to them for 23 h prior to all operant training and testing. Rats will be gradually acclimated to the water restriction schedule over several days by reducing the

amount of time each day that water is available in the home cage. Weight will be monitored to ensure that their body weight does not drop below 90% of their baseline, free water body weight. We will follow the rPVT training procedures used by others (Christie et al., 2008). Once trained, rPVT testing will consist of a stimulus light on for a duration of 0.5 s followed by a 3 s response period. The intertrial interval will vary between 3-7 s. Errors will result in a 10 s “time out” period during which the house lights will be turned off.

- **Surgical preparation of animals.** Mice will be surgically implanted for EEG/EMG recordings as described in our recent publications (Gerashchenko et al., 2008; Morairty et al., 2008; Wisor et al., 2008).
- **EEG data collection and analysis.** EEG/EMG recordings will be collected and analyzed as described in our recent publications (Gerashchenko et al., 2008; Morairty et al., 2008; Wisor et al., 2008).

▪ **Sleep deprivation.** SD procedures will be conducted as described previously (Gerashchenko et al., 2008; Wisor et al., 2008).

Immunohistochemistry. Sections will be treated for either Fos immunohistochemistry alone (single immunohistochemistry), or Fos immunohistochemistry followed by immunohistochemistry for one of the neurotransmitter markers (double immunohistochemistry). The following primary antisera were used for the neuronal markers: 1) to visualize cholinergic neurons, we will use rabbit anti-ChAT at 1:1000 dilution (AB143, Chemicon, Temecula, CA); 2) to nNOS neurons, we will use mouse anti-nNOS (1:5000; Sigma-Aldrich, St. Louis, MO); 3) to visualize histaminergic neurons, we will use rabbit-anti-adenosine deaminase (ADA, known to be co-expressed in histamine neurons in the rat tuberomammillary nucleus (Staines et al., 1987) at 1:1000 dilution (AB176, Chemicon); 4) to visualize serotonergic (5-HT) neurons, we will use rabbit-anti-serotonin at 1:5000 (AB125, Chemicon); 5) to visualize both dopaminergic (DA) and noradrenergic (NA) neurons, we will use rabbit-anti-tyrosine hydroxylase (TH) at 1:25,000 (AB151, Chemicon). Brain sections will be treated with 1% H₂O₂ for 15 min to quench endogenous peroxidases and then incubated overnight in rabbit-anti-cFos antisera (1:15,000, Calbiochem, San Diego, CA) at room temperature. Tissue will be then rinsed in PBS; incubated in biotinylated donkey anti-rabbit IgG (1:1000, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature; incubated with peroxidase-conjugated avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA) for 2 h, followed by addition of 0.05% diaminobenzidine tetrahydrochloride and 0.01% H₂O₂ with 1% NiSO₄ to produce a black reaction product in cell nuclei. Then, the sections will be incubated overnight in primary antibody. Tissue will be rinsed in PBS, incubated in biotinylated donkey anti-mouse or anti-rabbit IgG (1:500, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature, incubated with biotin-conjugated alkaline phosphatase (ABC-AP) for 2 h (Vector Laboratories, Burlingame, CA), washed again, and reacted in a working solution of Vector Red substrate (Vector Red Alkaline Phosphatase Substrate Kit I, Vector Laboratories, Burlingame, CA) to produce a red reaction product. All tissue will be mounted on gelatin-coated slides, dehydrated in ascending concentrations of ethanol, delipidated in xylenes, and coverslipped. Dilutions of all antibodies will be done in 5% donkey serum (Jackson ImmunoResearch, West Grove, PA), PBS, and 1% Triton X-100. The specificity of the nNOS monoclonal antibody has previously been established.

In vivo microdialysis. Rats will be anesthetized with isofluorane (2 to 3% in O₂) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). For surgical implantation of sleep recording electrodes under aseptic conditions, see above *Surgical preparation of animals*. Rats will also be unilaterally implanted with a stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO. BF coordinates relative to bregma are P -0.3, L +2.0, V -5.0 (Paxinos and Watson, 2005). The electrodes and guide cannula will be fixed using dental cement and a stainless steel skull screw to allow for microdialysis placement of the brain probe in freely moving rats. A dummy probe is inserted into the cannula to prevent occlusion prior to the onset of dialysis. For microdialysis, the dummy is removed and the microdialysis probe inserted and locked into

position such that the tip of the probe membrane extends 2.0 mm below the edge of the guide cannula. Rats will be given a 2 wk post-surgical recovery period prior to entering the experimental paradigm. The rats will then be conditioned to the microdialysis chamber and handled for at least 4 h every day for 1 wk prior to the onset of the experiment in order to limit the stress on the day of the experiment from exposure to a novel environment.

High-performance liquid chromatography (HPLC) analysis of adenosine. Immediately following collection, each dialysis sample will be injected into a HPLC/UV system (BASi, West Lafayette, IN) for quantification of ADO. A mobile phase consisting of 10 mM NaH_2PO_2 (pH 4.5) plus 9% of CH_3OH with a flow rate of 80 $\mu\text{l}/\text{min}$ is produced via a pump (PM-80, BASi). Separation occurs by a BAS microbore column (biophase octyl; 5 μm ; 250X4.6 mm; BASi) attached to the injector. A UV detector (wavelength = 254 nm; BASi) determines purine concentration. The chromatographic data is digitized and stored to disk using ChromGraph® software (BASi). Area under the chromatographic peak for each dialysis sample is compared to a standard curve generated from known ADO amounts in order to quantify ADO content in each sample. A standard curve is created prior to every experiment. Before each experiment, the probe is dialyzed with a known concentration of adenosine to determine the percentage of ADO recovered by the probe. Once the experiment is terminated, probe recovery of ADO is again quantified by dialyzing with the same known concentration of ADO. Percent recovery obtained before and after each experiment is compared by t-test. The purpose of this procedure is to ensure that probe membrane properties do not change during the course of the experiment, thus all experimental data reported will be from experiments that showed no statistically significant change in probe recovery.

In vitro electrophysiological recordings. At RT, hypothalamic slices will be transferred to a recording chamber (RC-27L, Warner Instrument Corp., CT) on a fluorescence microscope stage (Leica DM LFSA, Leica Instruments). Using a fluorescence microscope equipped with a high sensitivity, broad spectrum digital camera (CoolSNAP HQ2; contained in the **In Vitro Imaging System, JH Technologies, San Jose, CA**) for both infrared differential interference contrast (IR-DIC) imaging and fluorescent imaging, neurons with EGFP fluorescence will be identified as hypocretin neurons and used for electrophysiological recordings. Recordings will be carried out using a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and borosilicate pipettes (WPI, Sarasota, FL) prepared by a Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA). Micropipettes will be filled with intracellular solution (4–6 M Ohm) consisting of (in mM): 138 K-gluconate, 10 HEPES, 8 NaCl, 0.2 EGTA- Na_3 , 2 MgATP, 0.5 Na_2GTP , pH 7.3 with KOH. The osmolarity of the solution will be checked by a vapor pressure osmometer (model 5520, Wescor, Logan, UT). The osmolarity of the internal and external solutions will be 280–290 and 320–330 mOsm/L, respectively. Recording pipettes will be under positive pressure while advancing toward individual cells in the slice, and tight seals on the order of 1.0–1.5 G Ω will be made by negative pressure. The membrane patch will then be ruptured by suction. The series resistance during recording will be 10–25 M Ω . The reference electrode will be an Ag-AgCl pellet immersed in bath solution. During recordings at RT, cells will be superfused with extracellular solution at a rate of 1.6 mL/min using a peristaltic pump (Dynamax, Rainin, Oakland, CA). The output signal will be low pass filtered at 5 kHz and digitized at 10 kHz. Data will be recorded on a computer through a Digidata 1440 A/D converter using pClamp software (Molecular Devices, Sunnyvale, CA). For optogenetic stimulation in vitro, orange or blue light will be generated and controlled by a DG4 Wavelength Switcher contained in an **In Vitro Imaging System (JH Technologies, San Jose, CA)**.

In vivo extracellular recordings. The procedures to be used will be similar to those described in our recently published paper (Tsunematsu et al., 2011). Guided by a **surgical microscope (WPI Inc., Sarasota, FL)**, plastic fiber optics (0.5 mm diameter; Eska, Mitsubishi Rayon Co., Ltd., Tokyo, Japan) will be bilaterally implanted approximately 1 mm above the lateral hypothalamic area (1.7 mm posterior, 1 mm lateral from bregma, 4 mm depth) using a **4-axis micromanipulator (Siskiyou Instruments, Inc., Grants Pass, OR)** on a

vibration isolation table (TMC, Peabody, MA). Micropipettes will be prepared using a **Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA).** The activity of single neurons will be recorded extracellularly using glass pipette microelectrodes filled with a 0.5 M sodium acetate solution containing 2% Pontamine Sky Blue (PSB) (15–30 M Ω). The difference in electrical potential will be amplified (**Multiclamp 700B, Molecular Devices, Sunnyvale, CA**), filtered and digitized at a sampling rate of 20 kHz (**Digidata 1440, Molecular Devices, Sunnyvale, CA**) using **pClamp software (Molecular Devices, Sunnyvale, CA).** In Subtasks 6a and 6b, serotonergic neurons will be discriminated from others using the following criteria: (1) a longer duration of the action potential; (2) a shoulder on the falling phase or a deflection in the negative component of action potentials; and (3) the firing pattern during sleep/wakefulness (tonic firing during wakefulness, decreased firing frequency during SWS, and quiescence during REM sleep) (Takahashi et al., 2005). EEG and EMG signals will be amplified using an **electrophysiological data acquisition system (Tucker-Davis Tech, Alachua, FL)**, filtered and digitized at a sampling rate of 200 Hz (EEG) and 100 Hz (EMG). To mark the tip of the recording electrode by PSB labeling, current will be injected through the recording electrode (10 μ A for 6 min). After the experiment, the animal will be perfused sequentially with 20 mL chilled saline and 20 mL of chilled 10% formalin solution (Wako).

Optogenetic illumination *in vivo*. The procedures to be used will be similar to those described in our recently published paper (Tsunematsu et al., 2011). Continuous EEG and EMG recordings will be carried out through a slip ring designed so that the movement of the animal is unrestricted. EEG and EMG signals will be amplified using an **electrophysiological data acquisition system (Tucker-Davis Tech, Alachua, FL)**, filtered (EEG, 1.5–30 Hz; EMG, 15–300 Hz), digitized at a sampling rate of 250 Hz, and recorded using the software provided with **the data acquisition system (Tucker-Davis Tech, Alachua, FL).** Blue or orange light will be generated and controlled by an **Optogenetic LED Illumination system (Prizmatix Ltd., Modiin Ilite, Israel)** and applied through plastic optical fibers bilaterally inserted 1 mm above the LHA. Light power intensity at the tip of the plastic fiber optics (0.5 mm diameter) will be 0.4 mW/mm² measured by power meter (VEGA, Ophir Optronics Ltd., Wilmington, MA). The animal's behavior will be monitored through a CCD video camera and recorded on a computer synchronized with EEG and EMG recordings.

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